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### (54) METHODS FOR PRODUCING MODIFIED GLYCOPROTEINS

VERFAHREN FÜR DIE HERSTELLUNG MODIFIZierter GLYKOPROTEINE

PROCEDE DE PRODUCTION DE GLYCOPROTEINES MODIFIEES

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(73) Proprietor: **Glycofi, Inc.  
Lebanon, New Hampshire 03766 (US)**

(72) Inventor: **GERNGROSS, Tillman, U.  
Hanover, NH 03755 (US)**

(74) Representative: **Vossius & Partner  
Siebertstrasse 4  
81675 München (DE)**

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**Description****BACKGROUND OF THE INVENTION**Glycosylation Pathways

[0001] *De novo* synthesized proteins may undergo further processing in cells, known as post-translational modification. In particular, sugar residues may be added enzymatically, a process known as glycosylation. The resulting proteins bearing covalently linked oligosaccharide side chains are known as glycosylated proteins or glycoproteins. Bacteria typically do not glycosylate proteins; in cases where glycosylation does occur it usually occurs at nonspecific sites in the protein (Moens and Vanderleyden, Arch. Microbiol. 1997 168(3):169-175).

[0002] Eukaryotes commonly attach a specific oligosaccharide to the side chain of a protein asparagine residue, particularly an asparagine which occurs in the sequence Asn-Xaa-Ser/Thr/Cys (where Xaa represents any amino acid). Following attachment of the saccharide moiety, known as an *N*-glycan, further modifications may occur *in vivo*. Typically these modifications occur via an ordered sequence of enzymatic reactions, known as a cascade. Different organisms provide different glycosylation enzymes (glycosyltransferases and glycosidases) and different glycosyl substrates, so that the final composition of a sugar side chain may vary markedly depending upon the host.

[0003] For example, microorganisms such as filamentous fungi and yeast (lower eukaryotes) typically add additional mannose and/or mannosylphosphate sugars. The resulting glycan is known as a "high-mannose" type or a mannan. By contrast, in animal cells, the nascent oligosaccharide side chain may be trimmed to remove several mannose residues and elongated with additional sugar residues that typically do not occur in the *N*-glycans of lower eukaryotes. See R.K. Bretthauer, et al. *Biotechnology and Applied Biochemistry*, 1999, 30, 193-200; W. Martinet, et al. *Biotechnology Letters*, 1998, 20, 1171-1177; S. Weikert, et al. *Nature Biotechnology*, 1999, 17, 1116-1121; M. Malissard, et al. *Biochemical and Biophysical Research Communications*, 2000, 267, 169-173; Jarvis, et al. 1998 Engineering *N*-glycosylation pathways in the baculovirus-insect cell system, *Current Opinion in Biotechnology*, 9:528-533; and M. Takeuchi, 1997 *Trends in Glycoscience and Glycotechnology*, 1997, 9, S29-S35.

[0004] The *N*-glycans that are produced in humans and animals are commonly referred to as complex *N*-glycans. A complex *N*-glycan means a structure with typically two to six outer branches with a sialyllactosamine sequence linked to an inner core structure Man<sub>3</sub>GlcNAc<sub>2</sub>. A complex *N*-glycan has at least one branch, and preferably at least two, of alternating GlcNAc and galactose (Gal) residues that terminate in oligosaccharides such as, for example: NeuNAc-; NeuAcα2-6GalNAcα1-; NeuAcα2-3 Galβ1-3GalNAcα1-; NeuAcα2-3/6Galβ1-4GlcNAcβ1-; GlcNAcα1-4Galβ1-(mucins only); Fucα1-2Galβ1-(blood group H). Sulfate esters can occur on galactose, GalNAc, and GlcNAc residues, and phosphate esters can occur on mannose residues. NeuAc (Neu: neuraminic acid; Ac:acetyl) can be O-acetylated or replaced by NeuGl (*N*-glycolylneuraminic acid). Complex *N*-glycans may also have intrachain substitutions of bisecting GlcNAc and core fucose (Fuc).

[0005] Human glycosylation begins with a sequential set of reactions in the endoplasmatic reticulum (ER) leading to a core oligosaccharide structure, which is transferred onto *de novo* synthesized proteins at the asparagine residue in the sequence Asn-Xaa-Ser/Thr (see Figure 1A). Further processing by glucosidases and mannosidases occurs in the ER before the nascent glycoprotein is transferred to the early Golgi apparatus, where additional mannose residues are removed by Golgi-specific 1,2-mannosidases. Processing continues as the protein proceeds through the Golgi. In the medial Golgi a number of modifying enzymes including *N*-acetylglucosamine transferases (GnT I, GnT II, GnT III, GnT IV GnT V GnT VI), mannosidase II, fucosyltransferases add and remove specific sugar residues (see Figure 1B). Finally in the trans Golgi, the *N*-glycans are acted on by galactosyl transferases and sialyltransferases (ST) and the finished glycoprotein is released from the Golgi apparatus. The protein *N*-glycans of animal glycoproteins have bi-, tri-, or tetra-antennary structures, and may typically include galactose, fucose, and *N*-acetylglucosamine. Commonly the terminal residues of the *N*-glycans consist of sialic acid. A typical structure of a human *N*-glycan is shown in Figure 1B.

Sugar Nucleotide Precursors

[0006] The *N*-glycans of animal glycoproteins typically include galactose, fucose, and terminal sialic acid. These sugars are not generally found on glycoproteins produced in yeast and filamentous fungi. In humans, the full range of nucleotide sugar precursors (e.g. UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, CMP-*N*-acetylneuraminic acid, UDP-galactose, GDP-fucose etc.) are generally synthesized in the cytosol and transported into the Golgi, where they are attached to the core oligosaccharide by glycosyltransferases. (Sommers and Hirschberg, 1981 *J. Cell Biol.* 91(2): A406-A406; Sommers and Hirschberg 1982 *J. Biol. Chem.* 257(18): 811-817; Perez and Hirschberg 1987 *Methods in Enzymology* 138: 709-715).

[0007] Glycosyl transfer reactions typically yield a side product which is a nucleoside diphosphate or monophosphate. While monophosphates can be directly exported in exchange for nucleoside triphosphate sugars by an antiport mech-

anism, diphosphonucleosides (e.g. GDP) have to be cleaved by phosphatases (e.g. GDPase) to yield nucleoside monophosphates and inorganic phosphate prior to being exported. This reaction is important for efficient glycosylation; for example, GDPase from *S. cerevisiae* has been found to be necessary for mannosylation. However the GDPase has 90% reduced activity toward UDP (Berninsone et al., 1994 *J. Biol. Chem.* 269(1):207-211 $\alpha$ ). Lower eukaryotes typically lack UDP-specific diphosphatase activity in the Golgi since they do not utilize UDP-sugar precursors for Golgi-based glycoprotein synthesis. *Schizosaccharomyces pombe*, a yeast found to add galactose residues to cell wall polysaccharides (from UDP-galactose) has been found to have specific UDPase activity, indicating the requirement for such an enzyme (Berninsone et al., 1994). UDP is known to be a potent inhibitor of glycosyltransferases and the removal of this glycosylation side product is important in order to prevent glycosyltransferase inhibition in the lumen of the Golgi (Khatara et al., 1974). See Beminsone, P., et al. 1995. *J. Biol.Chem.*270(24): 14564-14567; Beaudet, L., et al. 1998 *Abc Transporters: Biochemical, Cellular, and Molecular Aspects.* 292: 397-413.

#### Compartmentalization of Glycosylation Enzymes

**[0008]** Glycosyltransferases and mannosidases line the inner (luminal) surface of the ER and Golgi apparatus and thereby provide a catalytic surface that allows for the sequential processing of glycoproteins as they proceed through the ER and Golgi network. The multiple compartments of the cis, medial, and trans Golgi and the trans Golgi Network (TGN), provide the different localities in which the ordered sequence of glycosylation reactions can take place. As a glycoprotein proceeds from synthesis in the ER to full maturation in the late Golgi or TGN, it is sequentially exposed to different glycosidases, mannosidases and glycosyltransferases such that a specific *N*-glycan structure may be synthesized. The enzymes typically include a catalytic domain, a stem region, a membrane spanning region and an N-terminal cytoplasmic tail. The latter three structural components are responsible for directing a glycosylation enzyme to the appropriate locus.

**[0009]** Localization sequences from one organism may function in other organisms. For example the membrane spanning region of  $\alpha$ -2,6-sialyltransferase ( $\alpha$ -2,6-ST) from rats, an enzyme known to localize in the rat trans Golgi, was shown to also localize a reporter gene (invertase) in the yeast Golgi (Schwientek, et al., 1995). However, the very same membrane spanning region as part of a full-length of  $\alpha$ -2,6-sialyltransferase was retained in the ER and not further transported to the Golgi of yeast (Krezdom et al., 1994). A full length GalT from humans was not even synthesized in yeast, despite demonstrably high transcription levels. On the other hand the transmembrane region of the same human GalT fused to an invertase reporter was able to direct localization to the yeast Golgi, albeit it at low production levels. Schwientek and co-workers have shown that fusing 28 amino acids of a yeast mannosyltransferase (Mnt1), a region containing an N-terminal cytoplasmic tail, a transmembrane region and eight amino acids of the stem region, to the catalytic domain of human GalT are sufficient for Golgi localization of an active GalT (Schwientek et al. 1995 *J. Biol. Chem.* 270(10):5483-5489). Other galactosyltransferases appear to rely on interactions with enzymes resident in particular organelles since after removal of their transmembrane region they are still able to localize properly.

**[0010]** Improper localization of a glycosylation enzyme may prevent proper functioning of the enzyme in the pathway. For example *Aspergillus nidulans*, which has numerous  $\alpha$ -1,2-mannosidases (Eades and Hintz, 2000 *Gene* 255(1): 25-34), does not add GlcNAc to Man<sub>5</sub>GlcNAc<sub>2</sub> when transformed with the rabbit GnT I gene, despite a high overall level of GnT I activity (Kalsner et al., 1995). GnT I, although actively expressed, may be incorrectly localized such that the enzyme is not in contact with both of its substrates: the nascent *N*-glycan of the glycoprotein and UDP-GlcNAc. Alternatively, the host organism may not provide an adequate level of UDP-GlcNAc in the Golgi.

#### Glycoproteins Used Therapeutically

**[0011]** A significant fraction of proteins isolated from humans or other animals are glycosylated. Among proteins used therapeutically, about 70% are glycosylated. If a therapeutic protein is produced in a microorganism host such as yeast, however, and is glycosylated utilizing the endogenous pathway, its therapeutic efficiency is typically greatly reduced. Such glycoproteins are typically immunogenic in humans and show a reduced half-life *in vivo* after administration (Takeuchi, 1997).

**[0012]** Specific receptors in humans and animals can recognize terminal mannose residues and promote the rapid clearance of the protein from the bloodstream. Additional adverse effects may include changes in protein folding, solubility, susceptibility to proteases, trafficking, transport, compartmentalization, secretion, recognition by other proteins or factors, antigenicity, or allergenicity. Accordingly, it has been necessary to produce therapeutic glycoproteins in animal host systems, so that the pattern of glycosylation is identical or at least similar to that in humans or in the intended recipient species. In most cases a mammalian host system, such as mammalian cell culture, is used.

Systems for Producing Therapeutic Glycoproteins

**[0013]** In order to produce therapeutic proteins that have appropriate glycoforms and have satisfactory therapeutic effects, animal or plant-based expression systems have been used. The available systems include:

1. Chinese hamster ovary cells (CHO), mouse fibroblast cells and mouse myeloma cells (Arzneimittelforschung. 1998 Aug;48(8):870-880);
2. transgenic animals such as goats, sheep, mice and others (Dente Prog. Clin. Biol. 1989 Res. 300:85-98, Ruther et al., 1988 Cell 53(6):847-856; Ware, J., et al. 1993 *Thrombosis and Haemostasis* 69(6): 1194-1194; Cole, E. S., et al. 1994 *J.Cell.Biochem.* 265-265);
3. plants (*Arabidopsis thaliana*, tobacco etc.) (Staub, et al. 2000 *Nature Biotechnology* 18(3): 333-338) (McGarvey, P. B., et al. 1995 *Bio Technology* 13(13): 1484-1487; Bardor, M., et al. 1999 *Trends in Plant Science* 4(9): 376-380);
4. insect cells (*Spodoptera frugiperda* Sf9, Sf21, *Trichoplusia ni*, etc. in combination with recombinant baculoviruses such as *Autographa californica* multiple nuclear polyhedrosis virus which infects lepidopteran cells) (Altmans et al., 1999 *Glycoconj. J.* 16(2):109-123).

**[0014]** Recombinant human proteins expressed in the above-mentioned host systems may still include non-human glycoforms (Raju et al., 2000 *Annals Biochem.* 283(2):123-132). In particular, fraction of the *N*-glycans may lack terminal sialic acid, typically found in human glycoproteins. Substantial efforts have been directed to developing processes to obtain glycoproteins that are as close as possible in structure to the human forms, or have other therapeutic advantages. Glycoproteins having specific glycoforms may be especially useful, for example in the targeting of therapeutic proteins. For example, the addition of one or more sialic acid residues to a glycan side chain may increase the lifetime of a therapeutic glycoprotein *in vivo* after administration. Accordingly, the mammalian host cells may be genetically engineered to increase the extent of terminal sialic acid in glycoproteins expressed in the cells. Alternatively sialic acid may be conjugated to the protein of interest *in vitro* prior to administration using a sialic acid transferase and an appropriate substrate. In addition, changes in growth medium composition or the expression of enzymes involved in human glycosylation have been employed to produce glycoproteins more closely resembling the human forms (S. Weikert, et al., *Nature Biotechnology*, 1999, 17, 1116-1121; Werner, Noe, et al 1998 *Arzneimittelforschung* 48(8):870-880; Weikert, Papac et al., 1999; Andersen and Goochee 1994 *Cur. Opin.Biotechnol.* 5: 546-549; Yang and Butler 2000 *Biotechnol. Bioengin.* 68(4): 370-380). Alternatively cultured human cells may be used.

**[0015]** However, all of the existing systems have significant drawbacks. Only certain therapeutic proteins are suitable for expression in animal or plant systems (e.g. those lacking in any cytotoxic effect or other effect adverse to growth). Animal and plant cell culture systems are usually very slow, frequently requiring over a week of growth under carefully controlled conditions to produce any useful quantity of the protein of interest. Protein yields nonetheless compare unfavorably with those from microbial fermentation processes. In addition cell culture systems typically require complex and expensive nutrients and cofactors, such as bovine fetal serum. Furthermore growth may be limited by programmed cell death (apoptosis).

**[0016]** Moreover, animal cells (particularly mammalian cells) are highly susceptible to viral infection or contamination. In some cases the virus or other infectious agent may compromise the growth of the culture, while in other cases the agent may be a human pathogen rendering the therapeutic protein product unfit for its intended use. Furthermore many cell culture processes require the use of complex, temperature-sensitive, animal-derived growth media components, which may carry pathogens such as bovine spongiform encephalopathy (BSE) prions. Such pathogens are difficult to detect and/or difficult to remove or sterilize without compromising the growth medium. In any case, use of animal cells to produce therapeutic proteins necessitates costly quality controls to assure product safety.

**[0017]** Transgenic animals may also be used for manufacturing high-volume therapeutic proteins such as human serum albumin, tissue plasminogen activator, monoclonal antibodies, hemoglobin, collagen, fibrinogen and others. While transgenic goats and other transgenic animals (mice, sheep, cows, etc.) can be genetically engineered to produce therapeutic proteins at high concentrations in the milk, the process is costly since every batch has to undergo rigorous quality control. Animals may host a variety of animal or human pathogens, including bacteria, viruses, fungi, and prions. In the case of scrapies and bovine spongiform encephalopathy, testing can take about a year to rule out infection. The production of therapeutic compounds is thus preferably carried out in a well-controlled sterile environment, e.g. under Good Manufacturing Practice (GMP) conditions. However, it is not generally feasible to maintain animals in such environments. Moreover, whereas cells grown in a fermenter are derived from one well characterized Master Cell Bank (MCB), transgenic animal technology relies on different animals and thus is inherently non-uniform. Furthermore external factors such as different food uptake, disease and lack of homogeneity within a herd, may effect glycosylation patterns of the final product. It is known in humans, for example, that different dietary habits result in differing glycosylation patterns.

**[0018]** Transgenic plants have been developed as a potential source to obtain proteins of therapeutic value. However,

high level expression of proteins in plants suffers from gene silencing, a mechanism by which the genes for highly expressed proteins are down-regulated in subsequent plant generations. In addition, plants add xylose and/or  $\alpha$ -1,3-linked fucose to protein *N*-glycans, resulting in glycoproteins that differ in structure from animals and are immunogenic in mammals (Altmann, Marz et al., 1995 Glycoconj. J. 12(2):150-155). Furthermore, it is generally not practical to grow plants in a sterile or GMP environment, and the recovery of proteins from plant tissues is more costly than the recovery from fermented microorganisms.

#### Glycoprotein Production Using Eukaryotic Microorganisms

**[0019]** The lack of a suitable expression system is thus a significant obstacle to the low-cost and safe production of recombinant human glycoproteins. Production of glycoproteins via the fermentation of microorganisms would offer numerous advantages over the existing systems. For example, fermentation-based processes may offer (a) rapid production of high concentrations of protein; (b) the ability to use sterile, well-controlled production conditions (e.g. GMP conditions); (c) the ability to use simple, chemically defined growth media; (d) ease of genetic manipulation; (e) the absence of contaminating human or animal pathogens; (f) the ability to express a wide variety of proteins, including those poorly expressed in cell culture owing to toxicity etc.; (g) ease of protein recovery (e.g. via secretion into the medium). In addition, fermentation facilities are generally far less costly to construct than cell culture facilities.

**[0020]** As noted above, however, bacteria, including species such as *Escherichia coli* commonly used to produce recombinant proteins, do not glycosylate proteins in a specific manner like eukaryotes. Various methylotrophic yeasts such as *Pichia pastoris*, *Pichia methanolica*, and *Hansenula polymorpha*, are particularly useful as eukaryotic expression systems, since they are able to grow to high cell densities and/or secrete large quantities of recombinant protein. However, as noted above, glycoproteins expressed in these eukaryotic microorganisms differ substantially in *N*-glycan structure from those in animals. This has prevented the use of yeast or filamentous fungi as hosts for the production of many useful glycoproteins.

**[0021]** Several efforts have been made to modify the glycosylation pathways of eukaryotic microorganisms to provide glycoproteins more suitable for use as mammalian therapeutic agents. For example, several glycosyltransferases have been separately cloned and expressed in *S. cerevisiae* (GalT, GnT I), *Aspergillus nidulans* (GnT I) and other fungi (Yoshida et al., 1999, Kalsner et al., 1995 Glycoconj. J. 12(3):360-370, Schwientek et al., 1995). However, *N*-glycans with human characteristics were not obtained.

**[0022]** Yeasts produce a variety of mannosyltransferases e.g. 1,3-mannosyltransferases (e.g. MNN1 in *S. cerevisiae*) (Graham and Emr, 1991 J. Cell. Biol. 114(2):207-218), 1,2-mannosyltransferases (e.g. KTR/KRE family from *S. cerevisiae*), 1,6-mannosyltransferases (OCH1 from *S. cerevisiae*), mannosylphosphate transferases (MNN4 and MNN6 from *S. cerevisiae*) and additional enzymes that are involved in endogenous glycosylation reactions. Many of these genes have been deleted individually, giving rise to viable organisms having altered glycosylation profiles. Examples are shown in Table 1.

Table 1.

Examples of yeast strains having altered mannosylation			
Strain	<i>N</i> -glycan (wild Mutation type)	<i>N</i> -glycan (mutant)	Reference
<i>S. pombe</i>	Man <sub>9</sub> GlcNAc <sub>2</sub> OCH1	Man <sub>8</sub> GlcNAc <sub>2</sub>	Yoko-o et al., 2001 FEBS Lett. 489(1): 75-80
<i>S. cerevisiae</i>	Man <sub>9</sub> GlcNAc <sub>2</sub> OCH1/MNN1	Man <sub>8</sub> GlcNAc <sub>2</sub>	Nakanishi-Shindo et al., 1993 J. Biol. Chem. 268(35):26338-26345
<i>S. cerevisiae</i>	Man <sub>9</sub> GlcNAc <sub>2</sub> OCH1/MNN1/MNN4	Man <sub>8</sub> GlcNAc <sub>2</sub>	Chiba et al., 1998 J. Biol. Chem. 273, 26298-26304

**[0023]** In addition, Japanese Patent Application Public No. 8-336387 discloses an *OCH1* mutant strain of *Pichia pastoris*. The *OCH1* gene encodes 1,6-mannosyltransferase, which adds a mannose to the glycan structure Man<sub>8</sub>GlcNAc<sub>2</sub> to yield Man<sub>9</sub>GlcNAc<sub>2</sub>. The Man<sub>9</sub>GlcNAc<sub>2</sub> structure is then a substrate for further mannosylation *in vivo*, leading to the hypermannosylated glycoproteins that are characteristic of yeasts and typically may have at least 30-40 mannose residue per *N*-glycan. In the *OCH1* mutant strain, proteins glycosylated with Man<sub>8</sub>GlcNAc<sub>2</sub> are accumulated and hypermannosylation does not occur. However, the structure Man<sub>8</sub>GlcNAc<sub>2</sub> is not a substrate for animal glycosylation enzymes, such as human UDP-GlcNAc transferase I, and accordingly the method is not useful for producing

proteins with human glycosylation patterns.

[0024] Martinet et al. (Biotechnol. Lett. 1998, 20(12), 1171-1177) reported the expression of  $\alpha$ -1,2-mannosidase from *Trichoderma reesei* in *P. pastoris*. Some mannose trimming from the N-glycans of a model protein was observed. However, the model protein had no N-glycans with the structure Man<sub>5</sub>GlcNAc<sub>2</sub>, which would be necessary as an intermediate for the generation of complex N-glycans. Accordingly the method is not useful for producing proteins with human or animal glycosylation patterns.

[0025] Similarly, Chiba et al. 1998 expressed  $\alpha$ -1,2-mannosidase from *Aspergillus saitoi* in the yeast *Saccharomyces cerevisiae*. A signal peptide sequence (His-Asp-Glu-Leu) was engineered into the exogenous mannosidase to promote retention in the endoplasmic reticulum. In addition, the yeast host was a mutant lacking three enzyme activities associated with hypermannosylation of proteins: 1, 6-mannosyltransferase (*OCH1*); 1,3-mannosyltransferase (*MNN1*); and mannosylphosphatetransferase (*MNN4*). The N-glycans of the triple mutant host thus consisted of the structure Man<sub>8</sub>GlcNAc<sub>2</sub>, rather than the high mannose forms found in wild-type *S. cerevisiae*. In the presence of the highly expressed engineered mannosidase, the N glycans of a model protein (carboxypeptidase Y) were trimmed to give a mixture consisting of 27 mole % Man<sub>5</sub>GlcNAc<sub>2</sub>, 22 mole % Man<sub>6</sub>GlcNAc<sub>2</sub>, 22 mole % Man<sub>7</sub>GlcNAc<sub>2</sub>, 29 mole % Man<sub>8</sub>GlcNAc<sub>2</sub>. Trimming of the endogenous cell wall glycoproteins was less efficient, only 10 mole % of the N glycans having the desired Man<sub>5</sub>GlcNAc<sub>2</sub> structure. Chiba's work is also reported in EP-A1 1 211 311. Although Chiba expressed in high copy a more than saturating amount of his mannosidase and obtained relatively poor yields, he suggests that even more enzyme be expressed to cure the problem of low yields. He never suggests that the enzyme to be targeted should be chosen to work optimally in the selected host cell at the subcellular location where it is to convert its substrate.

[0026] Since only the Man<sub>5</sub>GlcNAc<sub>2</sub> glycans would be susceptible to further enzymatic conversion to human glycoforms, the method is not efficient for the production of proteins having human glycosylation patterns. In proteins having a single N-glycosylation site, at least 73 mole % would have an incorrect structure. In proteins having two or three N-glycosylation sites, respectively at least 93 or 98 mole % would have an incorrect structure. Such low efficiencies of conversion are unsatisfactory for the production of therapeutic agents, particularly as the separation of proteins having different glycoforms is typically costly and difficult.

[0027] With the object of providing a more human-like glycoprotein derived from a fungal host, U. S. Patent No. 5,834,251 to Maras and Contreras discloses a method for producing a hybrid glycoprotein derived from *Trichoderma reesei*. A hybrid N-glycan has only mannose residues on the Man $\alpha$  1-6 arm of the core and one or two complex antennae on the Man $\alpha$  1-3 arm. While this structure has utility, the method has the disadvantage that numerous enzymatic steps must be performed *in vitro*, which is costly and time-consuming. Isolated enzymes are expensive to prepare and maintain,

may need unusual and costly substrates (e. g. UDP-GlcNAc), and are prone to loss of activity and/or proteolysis under the conditions of use.

[0028] It is therefore an object of the present invention to provide a system and methods for humanizing glycosylation of recombinant glycoproteins expressed in *Pichia pastoris* and other lower eukaryotes such as *Hansenula polymorpha*, *Pichia stiptis*, *Pichia methanolica*, *Pichia sp.*, *Kluyveromyces sp.*, *Candida albicans*, *Aspergillus nidulans*, and *Trichoderma reesei*.

## SUMMARY OF THE INVENTION

[0029] Cell lines having genetically modified glycosylation pathways that allow them to carry out a sequence of enzymatic reactions, which mimic the processing of glycoproteins in humans, have been developed. Recombinant proteins expressed in these engineered hosts yield glycoproteins more similar, if not substantially identical, to their human counterparts. The lower eukaryotes, which ordinarily produce high-mannose containing N-glycans, including unicellular and multicellular fungi such as *Pichia pastoris*, *Hansenula polymorpha*, *Pichia stiptis*, *Pichia methanolica*, *Pichia sp.*, *Kluyveromyces sp.*, *Candida albicans*, *Aspergillus nidulans*, and *Trichoderma reesei*, are modified to produce N-glycans such as Man<sub>5</sub>GlcNAc<sub>2</sub> or other structures along human glycosylation pathways. This is achieved using a combination of engineering and/or selection of strains which: do not express certain enzymes which create the undesirable complex structures characteristic of the fungal glycoproteins, which express exogenous enzymes selected either to have optimal activity under the conditions present in the fungi where activity is desired, or which are targeted to an organelle where optimal activity is achieved, and combinations thereof wherein the genetically engineered eukaryote expresses multiple exogenous enzymes required to produce "human-like" glycoproteins.

[0030] In a first embodiment, the invention relates to a host cell that is a unicellular or filamentous fungus that does not display  $\alpha$ -1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein, having in its endoplasmic reticulum (ER) or Golgi apparatus a hybrid enzyme selected to have optimal activity in the ER or Golgi of said host cell, so that said host cell is capable of forming 50 to 100 mole% Man<sub>5</sub>GlcNAc<sub>2</sub> on a substrate glycoprotein, the hybrid enzyme comprising:

- (a) an exogenous mannosidase catalytic domain having optimal activity in said ER or Golgi at a pH between 5.1 and 8.0; fused to
- (b) a cellular targeting signal peptide not normally associated with the catalytic domain of (a), wherein said cellular targeting signal peptide targets said exogenous mannosidase catalytic domain to said ER or Golgi apparatus.

**[0031]** Thus, the invention relates to a microorganism that is engineered to express an exogenous  $\alpha$ -1,2-mannosidase enzyme having an optimal pH between 5.1 and 8.0, preferably between 5.9 and 7.5. Thus, the exogenous enzyme is targeted to the endoplasmic reticulum or Golgi apparatus of the host organism, where it trims N-glycans such as  $\text{Man}_8\text{GlcNAc}_2$  to yield  $\text{Man}_5\text{GlcNAc}_2$ . The latter structure is useful because it is identical to a structure formed in mammals, especially humans; it is a substrate for further glycosylation reactions *in vivo* and/or *in vitro* that produce a finished N-glycan that is similar or identical to that formed in mammals, especially humans; and it is not a substrate for hypermannosylation reactions that occur *in vivo* in yeast and other microorganisms and that render a glycoprotein highly immunogenic in animals.

**[0032]** In a second embodiment, the invention relates to a method for producing the host cell disclosed herein, the method comprising the step of transforming said unicellular or filamentous fungus with a nucleic acid molecule encoding a hybrid enzyme selected to have optimal activity in the ER or Golgi of said host cell, so that said host cell is capable of forming 50 to 100 mole%  $\text{Man}_5\text{GlcNAc}_2$  on a substrate glycoprotein, the hybrid enzyme comprising:

- (a) an exogenous mannosidase catalytic domain having optimal activity in said ER or Golgi at a pH between 5.1 and 8.0; fused to
- (b) a cellular targeting signal peptide not normally associated with the catalytic domain of (a), wherein said cellular targeting signal peptide targets said exogenous catalytic domain to said ER or Golgi apparatus.

**[0033]** In a third embodiment, the invention relates to a method for producing a glycoprotein in a host cell that is a unicellular or filamentous fungus that does not display  $\alpha$ -1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein, the method comprising the steps of:

- (a) expressing said glycoprotein in a population of said host cells transformed with a DNA library comprising at least two different genetic constructs, at least one of which comprises a DNA fragment encoding a hybrid enzyme comprising:

- (i) a glycosylation enzyme catalytic domain having optimal activity in said ER or Golgi at a pH between 5.1 and 8.0; fused to
- (ii) a cellular targeting signal peptide not normally associated with the catalytic domain of (i), wherein said cellular targeting signal peptide targets the ER or Golgi apparatus of the host cell; and

- (b) selecting the host cell exhibiting a glycoprotein with a desired glycosylation pattern on said glycoprotein.

**[0034]** Thus, the glycosylation pathway of a eukaryotic microorganism is modified in this embodiment by (a) constructing a DNA library including at least two genes encoding exogenous glycosylation enzymes; (b) transforming the microorganism with the library to produce a genetically mixed population expressing at least two distinct exogenous glycosylation enzymes; (c) selecting from the population a microorganism having the desired glycosylation phenotype. In another embodiment, a DNA library includes chimeric genes each encoding a protein localization sequence and a catalytic activity related to glycosylation. Accordingly, in a fourth embodiment the invention provides a DNA library comprising genetic constructs, the constructs comprising a DNA fragment encoding a cellular targeting signal peptide which targets to the endoplasmic reticulum or Golgi apparatus ligated in-frame with a DNA fragment encoding a glycosylation enzyme or catalytically active fragment thereof having optimal activity in said ER or Golgi at a pH between 5.1 and 8.0, said DNA library including at least two DNA fragments encoding a cellular targeting signal peptide and at least two DNA fragments encoding exogenous glycosylation enzymes. Organisms modified using the method are useful for producing glycoproteins having a glycosylation pattern similar or identical to mammals, especially humans.

**[0035]** In a fifth embodiment, the glycosylation pathway is modified to express a sugar nucleotide transporter enzyme. In a preferred embodiment, a nucleotide diphosphatase enzyme is also expressed. The transporter and diphosphatase improve the efficiency of engineered glycosylation steps, by providing the appropriate substrates for the glycosylation enzymes in the appropriate compartments, reducing competitive product inhibition, and promoting the removal of nucleoside diphosphates.

**[0036]** Finally, the invention relates to a glycoprotein composition obtainable by the methods disclosed herein, comprising 50 to 100 mole % of  $\text{Man}_5\text{GlcNAc}_2$  converted by GnT I *in vivo* to  $\text{GlcNAcMan}_5\text{GlcNAc}_2$ , said glycoprotein lacking galactose, fucose and terminal sialic acid.

## DESCRIPTION OF THE FIGURES

[0037]

- 5 Figure 1A is a schematic diagram of typical fungal N-glycosylation pathway.  
Figure 1B is a schematic diagram of a typical human N-glycosylation pathway.

## DETAILED DESCRIPTION OF THE INVENTION

10 [0038] The methods and recombinant lower eukaryotic strains described herein are used to make "humanized glycoproteins". The recombinant lower eukaryotes are made by engineering lower eukaryotes which do not express one or more enzymes involved in production of high mannose structures to express the enzymes required to produce human-like sugars. As used herein, a lower eukaryote is a unicellular or filamentous fungus. As used herein, a "humanized glycoprotein" refers to a protein having attached thereto N-glycans including less than four mannose residues,  
15 and the synthetic intermediates (which are also useful and can be manipulated further *in vitro*) having at least five mannose residues. In a preferred embodiment, the glycoproteins produced in the recombinant lower eukaryotic strains contain at least 27 mole % of the Man<sub>5</sub>GlcNAc<sub>2</sub> intermediate. This is achieved by cloning in a better mannosidase, i.e., an enzyme selected to have optimal activity under the conditions present in the organisms at the site where proteins are glycosylated, or by targeting the enzyme to the organelle where activity is desired.

20 [0039] In a preferred embodiment, eukaryotic strains which do not express one or more enzymes involved in the production of high mannose structures are used. These strains can be engineered or one of the many such mutants already described in yeasts, including a hypermannosylation-minus (OCH1) mutant in *Pichia pastoris*.

[0040] The strains can be engineered one enzyme at a time, or a library of genes encoding potentially useful enzymes can be created, and those strains having enzymes with optimal activities or producing the most "human-like" glycoproteins, selected.

25 [0041] Lower eukaryotes that are able to produce glycoproteins having the attached N-glycan Man<sub>5</sub>GlcNAc<sub>2</sub> are particularly useful since (a) lacking a high degree of mannosylation (e.g. greater than 8 mannoses per N-glycan, or especially 30-40 mannoses), they show reduced immunogenicity in humans; and (b) the N-glycan is a substrate for further glycosylation reactions to form an even more human-like glycoform, e. g. by the action of GlcNAc transferase I to form GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub>. Man<sub>5</sub>GlcNAc<sub>2</sub> must be formed *in vivo* in a high yield, at least transiently, since all subsequent glycosylation reactions require Man<sub>5</sub>GlcNAc<sub>2</sub> or a derivative thereof. Accordingly, a yield is obtained of greater than 27 mole %, more preferably a yield of 50-100 mole % glycoproteins in which a high proportion of N-glycans have Man<sub>5</sub>GlcNAc<sub>2</sub>. Preferred host cells of the invention yield 50-100 mole % glycoproteins in which N glycans have Man<sub>5</sub>GlcNAc<sub>2</sub> that has been converted by GlcNAc transferase I *in vivo*. It is then possible to perform further glycosylation reactions *in vitro*, using for example the method of U. S. Patent No. 5,834,251 to Maras and Contreras. In a preferred  
35 embodiment, at least one further glycosylation reaction is performed *in vivo*. In a highly preferred embodiment thereof, active forms of further glycosylating enzymes are expressed in the endoplasmic reticulum and/or Golgi apparatus.

Host Microorganisms

40 [0042] Yeast and filamentous fungi have both been successfully used for the production of recombinant proteins, both intracellular and secreted (Cereghino, J. L. and J. M. Cregg 2000 *FEMS Microbiology Reviews* 24 (1): 45-66; Harkki, A., et al. 1989 *Bio-Technology* 7 (6): 596; Berka, R. M., et al. 1992 *Abstr. Papers Amer. Chem. Soc.* 203: 121-B1OT; Svetina, M., et al. 2000 *J. Biotechnol.* 76 (2-3): 245-251.

45 [0043] Although glycosylation in yeast and fungi is very different than in humans, some common elements are shared. The first step, the transfer of the core oligosaccharide structure to the nascent protein, is highly conserved in all eukaryotes including yeast, fungi, plants and humans (compare Figures 1A and 1B). Subsequent processing of the core oligosaccharide, however, differs significantly in yeast and involves the addition of several mannose sugars. This step is catalyzed by mannosyltransferases residing in the Golgi (e. g. OCH1, MNT1, MNN1, etc.), which sequentially add mannose sugars to the core oligosaccharide. The resulting structure is undesirable for the production of humanoid proteins and it is thus desirable to reduce or eliminate mannosyl transferase activity. Mutants of *S. cerevisiae*, deficient in mannosyl transferase activity (e.g. och1 or mnn9 mutants) have shown to be non-lethal and display a reduced mannose content in the oligosaccharide of yeast glycoproteins. Other oligosaccharide processing enzymes, such as mannosylphosphate transferase may also have to be eliminated depending on the host's particular endogenous glycosylation pattern. After reducing undesired endogenous glycosylation reactions the formation of complex N-glycans has to be engineered into the host system. This requires the stable expression of several enzymes and sugar-nucleotide transporters. Moreover, one has to locate these enzymes in a fashion such that a sequential processing of the maturing glycosylation structure is ensured.

Target Glycoproteins

[0044] The methods described herein are useful for producing glycoproteins, especially glycoproteins used therapeutically in humans. Such therapeutic proteins are typically administered by injection, orally, pulmonary, or other means.

[0045] Examples of suitable target glycoproteins include, without limitation: erythropoietin, cytokines such as interferon- $\alpha$ , interferon- $\beta$ , interferon- $\gamma$ , interferon- $\omega$ , and granulocyte-CSF, coagulation factors such as factor VIII, factor IX, and human protein C, soluble IgE receptor  $\alpha$ -chain, IgG, IgM, urokinase, chymase, and urea trypsin inhibitor, IGF-binding protein, epidermal growth factor, growth hormone-releasing factor, annexin V fusion protein, angiostatin, vascular endothelial growth factor-2, myeloid progenitor inhibitory factor-1, and osteoprotegerin.

Method for Producing Glycoproteins Comprising the N-glycan Man<sub>5</sub>GlcNAc<sub>2</sub>

[0046] The first step involves the selection or creation of a lower eukaryote that is able to produce a specific precursor structure of Man<sub>5</sub>GlcNAc<sub>2</sub>, which is able to accept *in vivo* GlcNAc by the action of a GlcNAc transferase I. This step requires the formation of a particular isomeric structure of Man<sub>5</sub>GlcNAc<sub>2</sub>. This structure has to be formed within the cell at a high yield (in excess of 30%) since all subsequent manipulations are contingent on the presence of this precursor. Man<sub>5</sub>GlcNAc<sub>2</sub> structures are necessary for complex N-glycan formation, however, their presence is by no means sufficient, since Man<sub>5</sub>GlcNAc<sub>2</sub> may occur in different isomeric forms, which may or may not serve as a substrate for GlcNAc transferase I. Most glycosylation reactions are not complete and thus a particular protein generally contains a range of different carbohydrate structures (i.e. glycoforms) on its surface. The mere presence of trace amounts (less than 5%) of a particular structure like Man<sub>5</sub>GlcNAc<sub>2</sub> is of little practical relevance. It is the formation of a particular, GlcNAc transferase I accepting intermediate (Structure I) in high yield (above 30%), which is required. The formation of this intermediate is necessary and subsequently allows for the *in vivo* synthesis of complex N-glycans.

[0047] One can select such lower eukaryotes from nature or alternatively genetically engineer existing fungi or other lower eukaryotes to provide the structure *in vivo*. No lower eukaryote has been shown to provide such structures *in vivo* in excess of 1.8% of the total N-glycans (Maras et al., 1997), so a genetically engineered organism is preferred. Methods such as those described in U.S. Patent No. 5,595,900, may be used to identify the absence or presence of particular glycosyltransferases, mannosidases and sugar nucleotide transporters in a target organism of interest.

Inactivation of Fungal Glycosylation Enzymes such as 1,6-mannosyltransferase

[0048] The method described herein may be used to engineer the glycosylation pattern of a wide range of lower eukaryotes (e.g. *Hansenula polymorpha*, *Pichia stiptis*, *Pichia methanolica*, *Pichia sp*, *Kluyveromyces sp*, *Candida albicans*, *Aspergillus nidulans*, *Trichoderma reesei* etc.). *Pichia pastoris* is used to exemplify the required manipulation steps. Similar to other lower eukaryotes, *P.pastoris* processes Man<sub>9</sub>GlcNAc<sub>2</sub> structures in the ER with a 1,2-  $\alpha$ - mannosidase to yield Man<sub>8</sub>GlcNAc<sub>2</sub>. Through the action of several mannosyltransferases, this structure is then converted to hypermannosylated structures (Man<sub>>9</sub>GlcNAc<sub>2</sub>), also known as mannans. In addition, it has been found that *P.pastoris* is able to add non-terminal phosphate groups, through the action of mannosylphosphate transferases to the carbohydrate structure. This is contrary to the reactions found in mammalian cells, which involve the removal of mannose sugars as opposed to their addition. It is of particular importance to eliminate the ability of the fungus to hypermannosylate the existing Man<sub>8</sub>GlcNAc<sub>2</sub> structure. This can be achieved by either selecting for a fungus that does not hypermannosylate, or by genetically engineering such a fungus.

[0049] Genes that are involved in this process have been identified in *Pichia pastoris* and by creating mutations in these genes one is able to reduce the production of "undesirable" glycoforms. Such genes can be identified by homology to existing mannosyltransferases (e.g. OCH1, MNN4, MNN6, MNN1), found in other lower eukaryotes such as *C. albicans*, *Pichia angusta* or *S. cerevisiae* or by mutagenizing the host strain and selecting for a phenotype with reduced mannosylation. Based on homologies amongst known mannosyltransferases and mannosylphosphate transferases, one may either design PCR primers, examples of which are shown in Table 2, or use genes or gene fragments encoding such enzymes as probes to identify homologues in DNA libraries of the target organism. Alternatively, one may be able to complement particular phenotypes in related organisms. For example, in order to obtain the gene or genes encoding 1,6-mannosyltransferase activity in *P.pastoris*, one would carry out the following steps. OCH1 mutants of *S. cerevisiae* are temperature sensitive and are slow growers at elevated temperatures. One can thus identify functional homologues of OCH1 in *P.pastoris* by complementing an OCH1 mutant of *S.cerevisiae* with a *P.pastoris* DNA or cDNA library. Such mutants of *S.cerevisiae* may be found at <http://genome-www.stanford.edu/Saccharomyces/> and are commercially available at <http://www.resgen.com/products/YEASTD.php3>. Mutants that display a normal growth phenotype at elevated temperature, after having been transformed with a *P.pastoris* DNA library, are likely to carry an OCH1 homologue of *P.pastoris*. Such a library can be created by partially digesting chromosomal DNA of *P.pastoris* with a suitable re-

striction enzyme and after inactivating the restriction enzyme ligating the digested DNA into a suitable vector, which has been digested with a compatible restriction enzyme. Suitable vectors are pRS314, a low copy (CEN6/ARS4) plasmid based on pBluescript containing the Trp1 marker (Sikorski, R S., and Hieter, P., 1989, Genetics 122, pg 19-27) or pFL44S, a high copy (2 $\mu$ ) plasmid based on a modified pUC19 containing the URA3 marker (Bonneaud, N., et al., 1991, Yeast 7, pg. 609-615). Such vectors are commonly used by academic researchers or similar vectors are available from a number of different vendors such as Invitrogen (Carlsbad, CA), Pharmacia (Piscataway, NJ), New England Biolabs (Beverly, MA). Examples are pYES/GS, 2 $\mu$  origin of replication based yeast expression plasmid from Invitrogen, or Yep24 cloning vehicle from New England Biolabs. After ligation of the chromosomal DNA and the vector one may transform the DNA library into strain of *S.cerevisiae* with a specific mutation and select for the correction of the corresponding phenotype. After sub-cloning and sequencing the DNA fragment that is able to restore the wild-type phenotype, one may use this fragment to eliminate the activity of the gene product encoded by OCH1 in *P. pastoris*.

**[0050]** Alternatively, if the entire genomic sequence of a particular fungus of interest is known, one may identify such genes simply by searching publicly available DNA databases, which are available from several sources such as NCBI, Swissprot etc. For example by searching a given genomic sequence or data base with a known 1,6 mannosyltransferase gene (OCH1) from *S. cerevisiae*, one is able to identify genes of high homology in such a genome, which a high degree of certainty encodes a gene that has 1,6 mannosyltransferase activity. Homologues to several known mannosyltransferases from *S.cerevisiae* in *P.pastoris* have been identified using either one of these approaches. These genes have similar functions to genes involved in the mannosylation of proteins in *S. cerevisiae* and thus their deletion may be used to manipulate the glycosylation pattern in *P.pastoris* or any other fungus with similar glycosylation pathways.

**[0051]** The creation of gene knock-outs, once a given target gene sequence has been determined, is a well-established technique in the yeast and fungal molecular biology community, and can be carried out by anyone of ordinary skill in the art (R. Rothsteins, (1991) Methods in Enzymology, vol. 194, p. 281). In fact, the choice of a host organism may be influenced by the availability of good transformation and gene disruption techniques for such a host. If several mannosyltransferases have to be knocked out, the method developed by Alani and Kleckner allows for the repeated use of the URA3 markers to sequentially eliminate all undesirable endogenous mannosyltransferase activity. This technique has been refined by others but basically involves the use of two repeated DNA sequences, flanking a counter selectable marker. For example: URA3 may be used as a marker to ensure the selection of transformants that have integrated a construct. By flanking the URA3 marker with direct repeats one may first select for transformants that have integrated the construct and have thus disrupted the target gene. After isolation of the transformants, and their characterization, one may counter select in a second round for those that are resistant to 5'FOA. Colonies that are able to survive on plates containing 5'FOA have lost the URA3 marker again through a crossover event involving the repeats mentioned earlier. This approach thus allows for the repeated use of the same marker and facilitates the disruption of multiple genes without requiring additional markers.

**[0052]** Eliminating specific mannosyltransferases, such as 1,6 mannosyltransferase (OCH1), mannosylphosphate transferases (MNN4, MNN6, or genes complementing *lbd* mutants) in *P. pastoris*, allows for the creation of engineered strains of this organism which synthesize primarily Man<sub>8</sub>GlcNAc<sub>2</sub> and thus can be used to further modify the glycosylation pattern to more closely resemble more complex human glycoform structures. A preferred embodiment of this method utilizes known DNA sequences, encoding known biochemical glycosylation activities to eliminate similar or identical biochemical functions in *P. pastoris*, such that the glycosylation structure of the resulting genetically altered *P. pastoris* strain is modified.

Table 2.

PCR primer A	PCR primer B	Target Gene(s) in <i>P. pastoris</i>	Homologues
ATGGCGAAGGCAGA TGGCAGT	TTAGTCCTTCCAAC TTCCTTC	1,6- mannosyltransferase	OCH1 <i>S.cerevisiae</i> , <i>Pichia albicans</i>
TAYTGGMGNGTNGA RCYNGAYATHAA	GCRTCNCCCCANCK YTCRTA	1,2 mannosyltransferase s	KTR/KRE family, <i>S.cerevisiae</i>

Legend: M = A or C, R = A or G, W = A or T, S = C or G,

Y = C or T, K = G or T, V = A or C or G, H = A or C or T, D =

A or G or T, B = C or G or T,

N = G or A or T or C.

#### Incorporation of a Mannosidase into the Genetically Engineered Host

**[0053]** The process described herein enables one to obtain such a structure in high yield for the purpose of modifying it to yield complex N-glycans. A successful scheme to obtain suitable  $\text{Man}_5\text{GlcNAc}_2$  structures must involve two parallel approaches: (1) reducing endogenous mannosyltransferase activity and (2) removing 1,2-  $\alpha$ - mannose by mannosidases to yield high levels of suitable  $\text{Man}_5\text{GlcNAc}_2$  structures. What distinguishes this method from the prior art is that it deals directly with those two issues. As the work of Chiba and coworkers demonstrates, one can reduce  $\text{Man}_8\text{GlcNAc}_2$  structures to a  $\text{Man}_5\text{GlcNAc}_2$  isomer in *S. cerevisiae*, by engineering the presence of a fungal mannosidase from *A. saitoi* into the ER. The shortcomings of their approach are twofold: (1) insufficient amounts of  $\text{Man}_5\text{GlcNAc}_2$  are formed in the extra-cellular glycoprotein fraction (10%) and (2) it is not clear that the *in vivo* formed  $\text{Man}_5\text{GlcNAc}_2$  structure in fact is able to accept GlcNAc by action of GlcNAc transferase I. If several glycosylation sites are present in a desired protein the probability (P) of obtaining such a protein in a correct form follows the relationship  $P=(F)^n$ , where n equals the number of glycosylation sites, and F equals the fraction of desired glycoforms. A glycoprotein with three glycosylation sites would have a 0.1 % chance of providing the appropriate precursors for complex and hybrid N-glycan processing on all of its glycosylation sites, which limits the commercial value of such an approach.

**[0054]** Most enzymes that are active in the ER and Golgi apparatus of *S.cerevisiae* have pH optima that are between 6.5 and 7.5 (see Table 3). All previous approaches to reduce mannosylation by the action of recombinant mannosidases have concentrated on enzymes that have a pH optimum around pH 5.0 (Martinet et al., 1998, and Chiba et al., 1998), even though the activity of these enzymes is reduced to less than 10% at pH 7.0 and thus most likely provide insufficient activity at their point of use, the ER and early Golgi of *P.pastoris* and *S.cenevisiae*. A preferred process utilizes an  $\alpha$ -mannosidase *in vivo*, where the pH optimum of the mannosidase is within 1.4 pH units of the average pH optimum of other representative marker enzymes localized in the same organelle(s). The pH optimum of the enzyme to be targeted to a specific organelle should be matched with the pH optimum of other enzymes found in the same organelle, such that the maximum activity per unit enzyme is obtained. Table 3 summarizes the activity of mannosidases from various sources and their respective pH optima. Table 4 summarizes their location.

Table 3.

Mannosidases and their pH optimum.			
Source	Enzyme	pH optimum	Reference
<i>Aspergillus saitoi</i>	1,2- $\alpha$ - mannosidase	5.0	Ichishima et al., 1999 Biochem. J. 339(Pt 3):589-597

Table 3. (continued)

Mannosidases and their pH optimum.			
Source	Enzyme	pH optimum	Reference
<i>Trichoderma reesei</i>	1,2- $\alpha$ - mannosidase	5.0	Maras et al., 2000 J. Biotechnol. 77 (2-3):255-263
<i>Penicillium citrinum</i>	1,2- $\alpha$ -D-mannosidase	5.0	Yoshida et al., 1993 Biochem. J. 290(Pt 2):349-354
<i>Aspergillus nidulans</i>	1,2- $\alpha$ - mannosidase	6.0	Eades and Hintz, 2000
<i>Homo sapiens</i> IA(Golgi)	1,2- $\alpha$ - mannosidase	6.0	
<i>Homo sapiens</i> IB (Golgi)	1,2- $\alpha$ - mannosidase	6.0	
<i>Lepidopteran</i> insect cells	Type I 1,2- $\alpha$ -Man <sub>6</sub> -mannosidase	6.0	Ren et al., 1995 Biochem. 34(8): 2489-2495
<i>Homo sapiens</i>	$\alpha$ - D-mannosidase	6.0	Chandrasekaran et al., 1984 Cancer Res. 44(9):4059-68
<i>Xanthomonas manihotis</i>	1,2,3- $\alpha$ - mannosidase	6.0	
<i>Mouse</i> IB (Golgi)	1,2- $\alpha$ - mannosidase	6.5	Schneikert and Herscovics, 1994 Glycobiology. 4(4):445-50
<i>Bacillus</i> sp. (secreted)	1,2- $\alpha$ -D-mannosidase	7.0	Maruyama et al., 1994 Carbohydrate Res. 251:89-98

**[0055]** When one attempts to trim high mannose structures to yield Man<sub>5</sub>GlcNAc<sub>2</sub> in the ER or the Golgi apparatus of *S. cerevisiae*, one may choose any enzyme or combination of enzymes that (1) has/have a sufficiently close pH optimum (i.e. between pH 5.2 and pH 7.8), and (2) is/are known to generate, alone or in concert, the specific isomeric Man<sub>5</sub>GlcNAc<sub>2</sub> structure required to accept subsequent addition of GlcNAc by GnT I. Any enzyme or combination of enzymes that has/have shown to generate a structure that can be converted to GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> by GnT I *in vitro* would constitute an appropriate choice. This knowledge may be obtained from the scientific literature or experimentally by determining that a potential mannosidase can convert Man<sub>8</sub>GlcNAc<sub>2</sub>-PA to Man<sub>5</sub>GlcNAc<sub>2</sub>-PA and then testing, if the obtained Man<sub>5</sub>GlcNAc<sub>2</sub>-PA structure can serve a substrate for GnT I and UDP-GlcNAc to give GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> *in vitro*. For example, mannosidase IA from a human or murine source would be an appropriate choice.

#### 1,2-Mannosidase Activity in the ER and Golgi

**[0056]** Previous approaches to reduce mannosylation by the action of cloned exogenous mannosidases have failed to yield glycoproteins having a sufficient fraction (e.g. >27 mole %) of *N*-glycans having the structure Man<sub>5</sub>GlcNAc<sub>2</sub> (Martinet et al., 1998, and Chiba et al., 1998). These enzymes should function efficiently in ER or Golgi apparatus to be effective in converting nascent glycoproteins. Whereas the two mannosidases utilized in the prior art (from *A. saitoi* and *T. reesei*) have pH optima of 5.0, most enzymes that are active in the ER and Golgi apparatus of yeast (e.g. *S. cerevisiae*) have pH optima that are between 6.5 and 7.5 (see Table 3). Since the glycosylation of proteins is a highly evolved and efficient process, it can be concluded that the internal pH of the ER and the Golgi is also in the range of about 6-8. At pH 7.0, the activity of the mannosidases used in the prior art is reduced to less than 10%, which is insufficient for the efficient production of Man<sub>5</sub>GlcNAc<sub>2</sub> *in vivo*. Chiba's work was also reported in EP-A1 1 211 310. Further comments are provided herein above.

Table 4.

Cellular location and pH optima of various glycosylation-related enzymes of <i>S. cerevisiae</i>				
Gene	Activity	Location	pH optimum	Author(s)
<i>Ktr1</i>	$\alpha$ - 1,2 mannosyltransferase	Golgi	7.0	Romero et al., 1997 Biochem. J. 321(Pt 2): 289-295
<i>Mns1</i>	$\alpha$ - 1,2- mannosidase	ER	6.5	
<i>CWH41</i>	glucosidase I	ER	6.8	

Table 4. (continued)

Cellular location and pH optima of various glycosylation-related enzymes of <i>S. cerevisiae</i>				
Gene	Activity	Location	pH optimum	Author(s)
--	mannosyltransferase	Golgi	7-8	Lehele and Tanner, 1974 Biochim. Biophys. Acta 350(1):225-235
<i>Kre2</i>	$\alpha$ -1,2 mannosyltransferase	Golgi	6.5-9.0	Romero et al., 1997

[0057] The  $\alpha$ -1,2-mannosidase enzyme should have optimal activity at a pH between 5.1 and 8.0. In a preferred embodiment, the enzyme has an optimal activity at a pH between 5.9 and 7.5. The optimal pH may be determined under *in vitro* assay conditions. Preferred mannosidases include those listed in Table 3 having appropriate pH optima, e.g. *Aspergillus nidulans*, *Homo sapiens* IA (Golgi), *Homo sapiens* IB (Golgi), *Lepidopteran* insect cells (IPLB-SF21AE), *Homo sapiens*, mouse IB (Golgi), and *Xanthomonas manihotis*. In a preferred embodiment, a single cloned mannosidase gene is expressed in the host organism. However, in some cases it may be desirable to express several different mannosidase genes, or several copies of one particular gene, in order to achieve adequate production of Man<sub>5</sub>GlcNAc<sub>2</sub>. In cases where multiple genes are used, the encoded mannosidases should all have pH optima within the preferred range of 5.1 to 8.0, or especially between 5.9 and 7.5. In an especially preferred embodiment mannosidase activity is targeted to the ER or cis Golgi, where the early reactions of glycosylation occur.

#### Formation of complex N-glycans

[0058] A second step of the process involves the sequential addition of sugars to the nascent carbohydrate structure by engineering the expression of glucosyltransferases into the Golgi apparatus. This process first requires the functional expression of GnT I in the early or medial Golgi apparatus as well as ensuring the sufficient supply of UDP-GlcNAc.

#### Integration Sites

[0059] Since the ultimate goal of this genetic engineering effort is a robust protein production strain that is able to perform well in an industrial fermentation process, the integration of multiple genes into the fungal chromosome involves careful planning. The engineered strain will most likely have to be transformed with a range of different genes, and these genes will have to be transformed in a stable fashion to ensure that the desired activity is maintained throughout the fermentation process. Any combination of the following enzyme activities will have to be engineered into the fungal protein expression host: sialyltransferases, mannosidases, fucosyltransferases, galactosyltransferases, glucosyltransferases, GlcNAc transferases, ER and Golgi specific transporters (e.g. syn and antiport transporters for UDP-galactose and other precursors), other enzymes involved in the processing of oligosaccharides, and enzymes involved in the synthesis of activated oligosaccharide precursors such as UDP-galactose, CMP-N-acetylneuraminic acid. At the same time a number of genes which encode enzymes known to be characteristic of non-human glycosylation reactions, will have to be deleted.

#### Targeting of glycosyltransferases to specific organelles:

[0060] Glycosyltransferases and mannosidases line the inner (luminal) surface of the ER and Golgi apparatus and thereby provide a "catalytic" surface that allows for the sequential processing of glycoproteins as they proceed through the ER and Golgi network. In fact the multiple compartments of the cis, medial, and trans Golgi and the trans-Golgi Network (TGN), provide the different localities in which the ordered sequence of glycosylation reactions can take place. As a glycoprotein proceeds from synthesis in the ER to full maturation in the late Golgi or TGN, it is sequentially exposed to different glycosidases, mannosidases and glycosyltransferases such that a specific carbohydrate structure may be synthesized. Much work has been dedicated to revealing the exact mechanism by which these enzymes are retained and anchored to their respective organelle. The evolving picture is complex but evidence suggests that stem region, membrane spanning region and cytoplasmic tail individually or in concert direct enzymes to the membrane of individual organelles and thereby localize the associated catalytic domain to that locus.

[0061] Targeting sequences are well known and described in the scientific literature and public databases, as discussed in more detail below with respect to libraries for selection of targeting sequences and targeted enzymes.

Method for Producing a Library to Produce Modified Glycosylation Pathways

**[0062]** A library including at least two genes encoding exogeneous glycosylation enzymes is transformed into the host organism, producing a genetically mixed population. Transformants having the desired glycosylation phenotypes are then selected from the mixed population. In a preferred embodiment, the host organism is a yeast, especially *P. pastoris*, and the host glycosylation pathway is modified by the operative expression of one or more human or animal glycosylation enzymes, yielding protein *N*-glycans similar or identical to human glycoforms. In an especially preferred embodiment, the DNA library includes genetic constructs encoding fusions of glycosylation enzymes with targeting sequences for various cellular loci involved in glycosylation especially the ER, cis Golgi, medial Golgi, or trans Golgi.

**[0063]** Examples of modifications to glycosylation which can be effected using method are: (1) engineering an eukaryotic microorganism to trim mannose residues from  $\text{Man}_8\text{GlcNAc}_2$  to yield  $\text{Man}_5\text{GlcNAc}_2$  as a protein *N*-glycan; (2) engineering an eukaryotic microorganism to add an *N*-acetylglucosamine (GlcNAc) residue to  $\text{Man}_5\text{GlcNAc}_2$  by action of GlcNAc transferase I; (3) engineering an eukaryotic microorganism to functionally express an enzyme such as an *N*-acetylglucosamine transferase (GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI), mannosidase II, fucosyltransferase, galactosyl transferase (GalT) or sialyltransferases (ST).

**[0064]** By repeating the method, increasingly complex glycosylation pathways can be engineered into the target microorganism. In one preferred embodiment, the host organism is transformed two or more times with DNA libraries including sequences encoding glycosylation activities. Selection of desired phenotypes may be performed after each round of transformation or alternatively after several transformations have occurred. Complex glycosylation pathways can be rapidly engineered in this manner.

*DNA Libraries*

**[0065]** It is necessary to assemble a DNA library including at least two exogenous genes encoding glycosylation enzymes. In addition to the open reading frame sequences, it is generally preferable to provide each library construct with such promoters, transcription terminators, enhancers, ribosome binding sites, and other functional sequences as may be necessary to ensure effective transcription and translation of the genes upon transformation into the host organism. Where the host is *Pichia pastoris*, suitable promoters include, for example, the *AOX1*, *AOX2*, *DAS*, and *P40* promoters. It is also preferable to provide each construct with at least one selectable marker, such as a gene to impart drug resistance or to complement a host metabolic lesion. The presence of the marker is useful in the subsequent selection of transformants; for example, in yeast the *URA3*, *HIS4*, *SUC2*, *G418*, *BLA*, or *SHBLE* genes may be used.

**[0066]** In some cases the library may be assembled directly from existing or wild-type genes. In a preferred embodiment however the DNA library is assembled from the fusion of two or more sub-libraries. By the in-frame ligation of the sub-libraries, it is possible to create a large number of novel genetic constructs encoding useful targeted glycosylation activities. For example, one useful sub-library includes DNA sequences encoding any combination of enzymes such as sialyltransferases, mannosidases, fucosyltransferases, galactosyltransferases, glucosyltransferases, and GlcNAc transferases. Preferably, the enzymes are of human origin, although other mammalian, animal, or fungal enzymes are also useful. In a preferred embodiment, genes are truncated to give fragments encoding the catalytic domains of the enzymes. By removing endogenous targeting sequences, the enzymes may then be redirected and expressed in other cellular loci. The choice of such catalytic domains may be guided by the knowledge of the particular environment in which the catalytic domain is subsequently to be active. For example, if a particular glycosylation enzyme is to be active in the late Golgi, and all known enzymes of the host organism in the late Golgi have a certain pH optimum, then a catalytic domain is chosen which exhibits adequate activity at that pH.

**[0067]** Another useful sub-library includes DNA sequences encoding signal peptides that result in localization of a protein to a particular location within the ER, Golgi, or trans Golgi network. These signal sequences may be selected from the host organism as well as from other related or unrelated organisms. Membrane-bound proteins of the ER or Golgi typically may include, for example, N-terminal sequences encoding a cytosolic tail (ct), a transmembrane domain (tmd), and a stem region (sr). The ct, tmd, and sr sequences are sufficient individually or in combination to anchor proteins to the inner (luminal) membrane of the organelle. Accordingly, a preferred embodiment of the sub-library of signal sequences includes ct, tmd, and/or sr sequences from these proteins. In some cases it is desirable to provide the sub-library with varying lengths of sr sequence. This may be accomplished by PCR using primers that bind to the 5' end of the DNA encoding the cytosolic region and employing a series of opposing primers that bind to various parts of the stem region. Still other useful sources of signal sequences include retrieval signal peptides, e.g. the tetrapeptides HDEL or KDEL, which are typically found at the C-terminus of proteins that are transported retrograde into the ER or Golgi. Still other sources of signal sequences include (a) type II membrane proteins, (b) the enzymes listed in Table 3, (c) membrane spanning nucleotide sugar transporters that are localized in the Golgi, and (d) sequences referenced in Table 5.

Table 5.

Sources of useful compartmental targeting sequences			
<i>Gene or Sequence</i>	<i>Organism</i>	<i>Function</i>	<i>Location of Gene Product</i>
<i>Mns1</i>	<i>S. cerevisiae</i>	$\alpha$ -1,2-mannosidase	ER
<i>OCH1</i>	<i>S. cerevisiae</i>	1,6-mannosyltransferase	Golgi (cis)
<i>MNN2</i>	<i>S. cerevisiae</i>	1,2-mannosyltransferase	Golgi (medial)
<i>MNN1</i>	<i>S. cerevisiae</i>	1,3-mannosyltransferase	Golgi (trans)
<i>OCH1</i>	<i>P. pastoris</i>	1,6-mannosyltransferase	Golgi (cis)
2,6 ST	<i>H. sapiens</i>	2,6-sialyltransferase	trans Golgi network
UDP-Gal T	<i>S. pombe</i>	UDP-Gal transporter	Golgi
<i>Mnt1</i>	<i>S. cerevisiae</i>	1,2-mannosyltransferase	Golgi (cis)
HDEL at C-terminus	<i>S. cerevisiae</i>	retrieval signal	ER

[0068] In any case, it is highly preferred that signal sequences are selected which are appropriate for the enzymatic activity or activities which are to be engineered into the host. For example, in developing a modified microorganism capable of terminal sialylation of nascent *N*-glycans, a process which occurs in the late Golgi in humans, it is desirable to utilize a sub-library of signal sequences derived from late Golgi proteins. Similarly, the trimming of Man<sub>8</sub>GlcNAc<sub>2</sub> by an  $\alpha$ -1,2-mannosidase to give Man<sub>5</sub>GlcNAc<sub>2</sub> is an early step in complex *N*-glycan formation in humans. It is therefore desirable to have this reaction occur in the ER or early Golgi of an engineered host microorganism. A sub-library encoding ER and early Golgi retention signals is used.

[0069] In a preferred embodiment, a DNA library is then constructed by the in-frame ligation of a sub-library including DNA encoding signal sequences with a sub-library including DNA encoding glycosylation enzymes or catalytically active fragments thereof. The resulting library includes synthetic genes encoding fusion proteins. In some cases it is desirable to provide a signal sequence at the N-terminus of a fusion protein, or in other cases at the C-terminus. In some cases signal sequences may be inserted within the open reading frame of an enzyme, provided the protein structure of individual folded domains is not disrupted.

[0070] The method is most effective when a DNA library transformed into the host contains a large diversity of sequences, thereby increasing the probability that at least one transformant will exhibit the desired phenotype. Accordingly, prior to transformation, a DNA library or a constituent sub-library may be subjected to one or more rounds of gene shuffling, error prone PCR, or *in vitro* mutagenesis.

#### Transformation

[0071] The DNA library is then transformed into the host organism. In yeast, any convenient method of DNA transfer may be used, such as electroporation, the lithium chloride method, or the spheroplast method. To produce a stable strain suitable for high-density fermentation, it is desirable to integrate the DNA library constructs into the host chromosome. In a preferred embodiment, integration occurs via homologous recombination, using techniques known in the art. For example, DNA library elements are provided with flanking sequences homologous to sequences of the host organism. In this manner integration occurs at a defined site in the host genome, without disruption of desirable or essential genes. In an especially preferred embodiment, library DNA is integrated into the site of an undesired gene in a host chromosome, effecting the disruption or deletion of the gene. For example, integration into the sites of the *OCH1*, *MNN1*, or *MNN4* genes allows the expression of the desired library DNA while preventing the expression of enzymes involved in yeast hypermannosylation of glycoproteins. In other embodiments, library DNA may be introduced into the host via a chromosome, plasmid, retroviral vector, or random integration into the host genome. In any case, it is generally desirable to include with each library DNA construct at least one selectable marker gene to allow ready selection of host organisms that have been stably transformed. Recyclable marker genes such as *ura3*, which can be selected for or against, are especially suitable.

#### Selection Process

[0072] After transformation of the host strain with the DNA library, transformants displaying the desired glycosylation phenotype are selected. Selection may be performed in a single step or by a series of phenotypic enrichment and/or depletion steps using any of a variety of assays or detection methods. Phenotypic characterization may be carried out

manually or using automated high-throughput screening equipment. Commonly a host microorganism displays protein *N*-glycans on the cell surface, where various glycoproteins are localized. Accordingly intact cells may be screened for a desired glycosylation phenotype by exposing the cells to a lectin or antibody that binds specifically to the desired *N*-glycan. A wide variety of oligosaccharide-specific lectins are available commercially (EY Laboratories, San Mateo, CA). Alternatively, antibodies to specific human or animal *N*-glycans are available commercially or may be produced using standard techniques. An appropriate lectin or antibody may be conjugated to a reporter molecule, such as a chromophore, fluorophore, radioisotope, or an enzyme having a chromogenic substrate (Guillen et al., 1998. *Proc. Natl. Acad. Sci. USA* 95(14): 7888-7892). Screening may then be performed using analytical methods such as spectrophotometry, fluorimetry, fluorescence activated cell sorting, or scintillation counting. In other cases, it may be necessary to analyze isolated glycoproteins or *N*-glycans from transformed cells. Protein isolation may be carried out by techniques known in the art. In cases where an isolated *N*-glycan is required, an enzyme such as endo- $\beta$ -*N*-acetylglucosaminidase (Genzyme Co., Boston, MA) may be used to cleave the *N*-glycans from glycoproteins. Isolated proteins or *N*-glycans may then be analyzed by liquid chromatography (e.g. HPLC), mass spectroscopy, or other suitable means. U.S. Patent No. 5,595,900 teaches several methods by which cells with desired extracellular carbohydrate structures may be identified. Prior to selection of a desired transformant, it may be desirable to deplete the transformed population of cells having undesired phenotypes. For example, when the method is used to engineer a functional mannosidase activity into cells, the desired transformants will have lower levels of mannose in cellular glycoprotein. Exposing the transformed population to a lethal radioisotope of mannose in the medium depletes the population of transformants having the undesired phenotype, i.e. high levels of incorporated mannose. Alternatively, a cytotoxic lectin or antibody, directed against an undesirable *N*-glycan, may be used to deplete a transformed population of undesired phenotypes.

#### Methods for Providing Sugar Nucleotide Precursors to the Golgi Apparatus

**[0073]** For a glycosyltransferase to function satisfactorily in the Golgi, it is necessary for the enzyme to be provided with a sufficient concentration of an appropriate nucleotide sugar, which is the high-energy donor of the sugar moiety added to a nascent glycoprotein. These nucleotide sugars to the appropriate compartments are provided by expressing an exogenous gene encoding a sugar nucleotide transporter in the host microorganism. The choice of transporter enzyme is influenced by the nature of the exogenous glycosyltransferase being used. For example, a GlcNAc transferase may require a UDP-GlcNAc transporter, a fucosyltransferase may require a GDP-fucose transporter, a galactosyltransferase may require a UDP-galactose transporter, or a sialyltransferase may require a CMP-sialic acid transporter.

**[0074]** The added transporter protein conveys a nucleotide sugar from the cytosol into the Golgi apparatus, where the nucleotide sugar may be reacted by the glycosyltransferase, e.g. to elongate an *N*-glycan. The reaction liberates a nucleoside diphosphate or monophosphate, e.g. UDP, GDP, or CMP. As accumulation of a nucleoside diphosphate inhibits the further activity of a glycosyltransferase, it is frequently also desirable to provide an expressed copy of a gene encoding a nucleotide diphosphatase. The diphosphatase (specific for UDP or GDP as appropriate) hydrolyzes the diphosphonucleoside to yield a nucleoside monophosphate and inorganic phosphate. The nucleoside monophosphate does not inhibit the glycotransferase and in any case is exported from the Golgi by an endogenous cellular system. Suitable transporter enzymes, which are typically of mammalian origin, are described below.

#### *Examples*

**[0075]** The use of the above general method may be understood by reference to the following non-limiting examples. Examples of preferred embodiments are also summarized in Table 6.

#### **Example 1: Engineering of *P. pastoris* with $\alpha$ -1,2-Mannosidase to produce interferon**

**[0076]** An  $\alpha$ -1,2-mannosidase is required for the trimming of  $\text{Man}_8\text{GlcNAc}_2$  to yield  $\text{Man}_5\text{GlcNAc}_2$ , an essential intermediate for complex *N*-glycan formation. An *OCH1* mutant of *P. pastoris* is engineered to express secreted human interferon- $\beta$  under the control of an *aox* promoter. A DNA library is constructed by the in-frame ligation of the catalytic domain of human mannosidase IB (an  $\alpha$ -1,2-mannosidase) with a sub-library including sequences encoding early Golgi localization peptides. The DNA library is then transformed into the host organism, resulting in a genetically mixed population wherein individual transformants each express interferon- $\beta$  as well as a synthetic mannosidase gene from the library. Individual transformant colonies are cultured and the production of interferon is induced by addition of methanol. Under these conditions, over 90.% of the secreted protein includes interferon- $\beta$ . Supernatants are purified to remove salts and low-molecular weight contaminants by  $\text{C}_{18}$  silica reversed-phase chromatography. Desired transformants expressing appropriately targeted, active  $\alpha$ -1,2-mannosidase produce interferon- $\beta$  including *N*-glycans of the structure  $\text{Man}_5\text{GlcNAc}_2$ , which has a reduced molecular mass compared to the interferon of the parent strain. The

purified supernatants including interferon- $\beta$  are analyzed by MALDI-TOF mass spectroscopy and colonies expressing the desired form of interferon- $\beta$  are identified.

#### Example 2: Engineering of Strain to express GlcNAc Transferase I

[0077] GlcNAc Transferase I activity is required for the maturation of complex *N*-glycans. Man<sub>5</sub>GlcNAc<sub>2</sub> may only be trimmed by mannosidase II, a necessary step in the formation of human glycoforms, after the addition of GlcNAc to the terminal  $\alpha$ -1,3 mannose residue by GlcNAc Transferase I (Schachter, 1991 Glycobiology 1(5):453-461). Accordingly a library is prepared including DNA fragments encoding suitably targeted GlcNAc Transferase I genes. The host organism is a strain, e.g. a yeast, that is deficient in hypermannosylation (e.g. an *OCH1* mutant), provides the substrate UDP-GlcNAc in the Golgi and/or ER, and provides *N*-glycans of the structure Man<sub>5</sub>GlcNAc<sub>2</sub> in the Golgi and/or ER. After transformation of the host with the DNA library, the transformants are screened for those having the highest concentration of terminal GlcNAc on the cell surface, or alternatively secrete the protein having the highest terminal GlcNAc content. Such a screen is performed using a visual method (e.g. a staining procedure), a specific terminal GlcNAc binding antibody, or a lectin. Alternatively the desired transformants exhibit reduced binding of certain lectins specific for terminal mannose residues.

#### Example 3: Engineering of Strains with a Mannosidase II

[0078] In another example, it is desirable in order to generate a human glycoform in a microorganism to remove the two remaining terminal mannoses from the structure GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> by action of a mannosidase II. A DNA library including sequences encoding cis and medial Golgi localization signals is fused in-frame to a library encoding mannosidase II catalytic domains. The host organism is a strain, e.g. a yeast, that is deficient in hypermannosylation (e.g. an *OCH1* mutant) and provides *N*-glycans having the structure GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub> in the Golgi and/or ER. After transformation, organisms having the desired glycosylation phenotype are selected. An *in vitro* assay is used in one method. The desired structure GlcNAcMan<sub>3</sub>GlcNAc<sub>2</sub> (but not the undesired GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub>) is a substrate for the enzyme GlcNAc Transferase II. Accordingly, single colonies may be assayed using this enzyme *in vitro* in the presence of the substrate, UDP-GlcNAc. The release of UDP is determined either by HPLC or an enzymatic assay for UDP. Alternatively radioactively labeled UDP-GlcNAc is used.

[0079] The foregoing *in vitro* assays are conveniently performed on individual colonies using high-throughput screening equipment. Alternatively a lectin binding assay is used. In this case the reduced binding of lectins specific for terminal mannoses allows the selection of transformants having the desired phenotype. For example, *Galantus nivalis* lectin binds specifically to terminal  $\alpha$ -1,3-mannose, the concentration of which is reduced in the presence of operatively expressed mannosidase II activity. In one suitable method, *G. nivalis* lectin attached to a solid agarose support (available from Sigma Chemical, St. Louis, MO) is used to deplete the transformed population of cells having high levels of terminal  $\alpha$ -1,3-mannose.

#### Example 4: Engineering of organisms to express Sialyltransferase

[0080] The enzymes  $\alpha$ 2,3-sialyltransferase and  $\alpha$ 2,6-sialyltransferase add terminal sialic acid to galactose residues in nascent human *N*-glycans, leading to mature glycoproteins. In human the reactions occur in the trans Golgi or TGN. Accordingly a DNA library is constructed by the in-frame fusion of sequences encoding sialyltransferase catalytic domains with sequences encoding trans Golgi or TGN localization signals. The host organism is a strain, e.g. a yeast, that is deficient in hypermannosylation (e.g. an *OCH1* mutant), which provides *N*-glycans having terminal galactose residues in the trans Golgi or TGN, and provides a sufficient concentration of CMP-sialic acid in the trans Golgi or TGN. Following transformation, transformants having the desired phenotype are selected using a fluorescent antibody specific for *N*-glycans having a terminal sialic acid.

#### Example 5: Method of engineering strains to express UDP-GlcNAc Transporter

[0081] The cDNA of human Golgi UDP-GlcNAc transporter has been cloned by Ishida and coworkers. (Ishida, N., et al. 1999 *J. Biochem.* 126(1): 68-77). Guillen and coworkers have cloned the canine kidney Golgi UDP-GlcNAc transporter by phenotypic correction of a *Kluyveromyces lactis* mutant deficient in Golgi UDP-GlcNAc transport. (Guillen, E., et al. 1998). Thus a mammalian Golgi UDP-GlcNAc transporter gene has all of the necessary information for the protein to be expressed and targeted functionally to the Golgi apparatus of yeast.

**Example 6: Method of engineering strains to express GDP-Fucose Transporter.**

[0082] The rat liver Golgi membrane GDP-fucose transporter has been identified and purified by Puglielli, L. and C. B. Hirschberg 1999 *J. Biol. Chem.* 274(50):35596-35600. The corresponding gene can be identified using standard techniques, such as N-terminal sequencing and Southern blotting using a degenerate DNA probe. The intact gene can then be expressed in a host microorganism that also expresses a fucosyltransferase.

**Example 7: Method of engineering strains to express UDP-Galactose Transporter**

[0083] Human UDP-galactose (UDP-Gal) transporter has been cloned and shown to be active in *S. cerevisiae*. (Kainuma, M., et al. 1999 *Glycobiology* 9(2): 133-141). A second human UDP-galactose transporter (hUGT1) has been cloned and functionally expressed in Chinese Hamster Ovary Cells. Aoki, K., et al. 1999 *J. Biochem.* 126(5): 940-950. Likewise Segawa and coworkers have cloned a UDP-galactose transporter from *Schizosaccharomyces pombe* (Segawa, H., et al. 1999 *Febs Letters* 451(3): 295-298).

**CMP-Sialic Acid Transporter**

[0084] Human CMP-sialic acid transporter (hCST) has been cloned and expressed in Lec 8 CHO cells by Aoki and coworkers (1999). Molecular cloning of the hamster CMP-sialic acid transporter has also been achieved (Eckhardt and Gerardy Schahn 1997 *Eur. J. Biochem.* 248(1): 187-192). The functional expression of the murine CMP-sialic acid transporter was achieved in *Saccharomyces cerevisiae* by Berninsone, P., et al. 1997 *J. Biol. Chem.* 272(19): 12616-12619.

Table 6.

Examples of preferred embodiments of the methods for modifying glycosylation in a eukaryotic microorganism, e. g. <i>Pichia pastoris</i>				
Desired Structure	Suitable Catalytic Activities	Suitable Sources of Localization Sequences	Suitable Gene Deletions	Suitable Transporters and/or Phosphatases
Man <sub>5</sub> GlcNAc <sub>2</sub>	α-1,2-mannosidase (murine, human, <i>Bacillus</i> sp., <i>A. nidulans</i> )	Mns1 (N-terminus, <i>S. cerevisiae</i> ) Och1 (N-terminus, <i>S. cerevisiae</i> , <i>P. pastoris</i> ) Ktr1 Mnn9 Mnt1 ( <i>S. cerevisiae</i> ) KDEL, HDEL (C-terminus)	OCH1 MNN4 MNN6	none
GlcNAcMan <sub>5</sub> GlcNAc <sub>2</sub>	GlcNAc Transferase I, (human, murine, rat etc.)	Och1 (N-terminus, <i>S. cerevisiae</i> , <i>P. pastoris</i> ) KTR1 (N-terminus) KDEL, HDEL (C-terminus) Mnn1 (N-terminus, <i>S. cerevisiae</i> ) Mnt1 (N-terminus, <i>S. cerevisiae</i> ) GDPase (N-terminus, <i>S. cerevisiae</i> )	OCH1 MNN4 MNN6	UDP-GlcNAc transporter (human, murine, <i>K. lactis</i> ) UDPase (human)

Table 6. (continued)

Examples of preferred embodiments of the methods for modifying glycosylation in a eukaryotic microorganism, e. g. <i>Pichia pastoris</i>				
Desired Structure	Suitable Catalytic Activities	Suitable Sources of Localization Sequences	Suitable Gene Deletions	Suitable Transporters and/or Phosphatases
GlcNAcMan <sub>3</sub> GlcNAc <sub>2</sub>	mannosidase II	Ktr1 Mnn1 (N-terminus, <i>S. cerevisiae</i> ) Mnt1 (N-terminus, <i>S. cerevisiae</i> ) Kre2/Mnt1 ( <i>S. cerevisiae</i> ) Kre2 ( <i>P. pastoris</i> ) Ktr1 ( <i>S. cerevisiae</i> ) Ktr1 ( <i>P. pastoris</i> ) Mnn1 ( <i>S. cerevisiae</i> )	OCH1 MNN4 MNN6	UDP-GlcNAc transporter (human, murine, <i>K. lactis</i> ) UDPase (human)
GlcNAc <sub>(2-4)</sub> Man <sub>3</sub> GlcNAc <sub>2</sub>	GlcNAcTransferase II, III, IV, V (human, murine)	Mnn1 (N-terminus, <i>S. cerevisiae</i> ) Mnt1 (N-terminus, <i>S. cerevisiae</i> ) Kre2/Mnt1 ( <i>S. cerevisiae</i> ) Kre2 ( <i>P. pastoris</i> ) Ktr1 ( <i>S. cerevisiae</i> ) Ktr1 ( <i>P. pastoris</i> ) Mnn1 ( <i>S. cerevisiae</i> )	OCH1 MNN4 MNN6	UDP-GlcNAc transporter (human, murine, <i>K. lactis</i> ) UDPase (human)
Gal <sub>(1-4)</sub> GlcNAc <sub>(2-4)</sub> Man <sub>3</sub> GlcNAc <sub>2</sub>	β-1,4-Galactosyl transferase (human)	Mnn1 (N-terminus, <i>S. cerevisiae</i> ) Mnt1 (N-terminus, <i>S. cerevisiae</i> ) Kre2/Mnt1 ( <i>S. cerevisiae</i> ) Kre2 ( <i>P. pastoris</i> ) Ktr1 ( <i>S. cerevisiae</i> ) Ktr1 ( <i>P. pastoris</i> ) Mnn1 ( <i>S. cerevisiae</i> )	OCH1 MNN4 MNN6	UDP-Galactose transporter (human, <i>S. pombe</i> )
NANA <sub>(1-4)</sub> Gal <sub>(1-4)</sub> GlcNAc <sub>(2-4)</sub> Man <sub>3</sub> GlcNAc <sub>2</sub>	α-2,6-Sialyltransferase (human) α-2,3-Sialyltransferase	KTR1 MNN1 (N-terminus, <i>S. cerevisiae</i> ) MNT1 (N-terminus, <i>S. cerevisiae</i> ) Kre2/Mnt1 ( <i>S. cerevisiae</i> ) Kre2 ( <i>P. pastoris</i> ) Ktr1 ( <i>S. cerevisiae</i> ) Ktr1 ( <i>P. pastoris</i> ) MNN1 ( <i>S. cerevisiae</i> )	OCH1 MNN4 MNN6	CMP-Sialic acid transporter (human)

Table 7:

DNA and Protein Sequence Resources

1. European Bioinformatics Institute (EBI) is a centre for research and services in bioinformatics: <http://www.ebi.ac.uk/>

Table 7: (continued)

## DNA and Protein Sequence Resources

2. Swissprot database: <http://www.expasy.ch/spr>

3. List of known glycosyltransferases and their origin.

 $\beta$ 1,2 (GnT I) EC 2.4.1.101

4. human cDNA, Kumar et al (1990) Proc. Natl. Acad. Sci. USA 87:9948-9952

5. human gene, Hull et al (1991) Biochem. Biophys. Res. Commun. 176:608-615

6. mouse cDNA, Kumar et al (1992) Glycobiology 2:383-393

7. mouse gene, Pownall et al (1992) Genomics 12:699-704

8. murine gene (5' flanking, non-coding), Yang et al (1994) Glycobiology 5:703-712

9. rabbit cDNA, Sarkar et al (1991) Proc. Natl. Acad. Sci. USA 88:234-238

10. rat cDNA, Fukada et al (1994) Biosci. Biotechnol. Biochem. 58:200-201

1,2 (GnT II) EC 2.4.1.143

11. human gene, Tan et al (1995) Eur. J. Biochem. 231:317-328

12. rat cDNA, D'Agostaro et al (1995) J. Biol. Chem. 270:15211-15221

13.  $\beta$ 1,4 (GnT III) EC 2.4.1.144

14. human cDNA, Ihara et al (1993) J. Biochem. 113:692-698

15. murine gene, Bhaumik et al (1995) Gene 164:295-300

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Table 7: (continued)

## DNA and Protein Sequence Resources

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**[0085]** Additional methods and reagents which can be used in the methods for modifying the glycosylation are described in the literature, such as U.S. Patent No. 5,955,422, U.S. Patent No. 4,775,622, U.S. Patent No. 6,017,743, U.S. Patent No. 4,925,796, U.S. Patent No. 5,766,910, U.S. Patent No. 5,834,251, U.S. Patent No. 5,910,570, U.S. Patent No. 5,849,904, U.S. Patent No. 5,955,347, U.S. Patent No. 5,962,294, U.S. Patent No. 5,135,854, U.S. Patent No. 4,935,349, U.S. Patent No. 5,707,828, and U.S. Patent No. 5,047,335.

**[0086]** Appropriate yeast expression systems can be obtained from sources such as the American Type Culture Collection, Rockville, MD. Vectors are commercially available from a variety of sources.

## Claims

1. A host cell that is a unicellular or filamentous fungus that does not display alpha-1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein, having in its endoplasmic reticulum (ER) or Golgi apparatus a hybrid enzyme selected to have optimal activity in the ER or Golgi of said host cell, so that said host cell is capable of forming 50 to 100 Mole% Man<sub>5</sub>GlcNAc<sub>2</sub> on a substrate glycoprotein, the hybrid enzyme comprising:

(a) an exogenous mannosidase catalytic domain having optimal activity in said ER or Golgi at a pH between 5.1 and 8.0; fused to

(b) a cellular targeting signal peptide not normally associated with the catalytic domain of (a), wherein said cellular targeting signal peptide targets said exogenous mannosidase catalytic domain to said ER or Golgi apparatus.

2. The host cell of claim 1, wherein said catalytic domain has  $\alpha$ -1,2-mannosidase activity.

3. The host cell of claim 1 or 2, wherein said mannosidase comprises an  $\alpha$ -1,2-mannosidase catalytic domain derived from mouse, human, *Lepidoptera*, *Aspergillus nidulans*, *Xanthomonas manihotis*, or *Bacillus* sp.

4. The host cell of any one of claims 1 to 3, wherein the mannosidase has optimal activity at a pH between 5.9 and 7.5.

5. The host cell of any one of claims 1 to 4, further expressing one or more exogenous enzymes selected from the group consisting of mannosidases, glycosyltransferases and glycosidases.

6. The host cell of claim 5, wherein at least one additional exogenous enzyme for the production of a glycoprotein is localized by forming a fusion protein between a catalytic domain of the exogenous enzyme and a cellular targeting signal peptide not normally associated with the catalytic domain that targets it to the endoplasmic reticulum, the early, medial or late Golgi, or the trans Golgi network of the host cell.

7. The host cell of claim 6, wherein said catalytic domain is from a glycosidase or glycosyltransferase selected from GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI.

8. The host cell of any one of claims 5 to 7, further expressing one or more exogenous enzymes selected from the group consisting of UDP-GlcNAc transferase, UDP-GlcNAc transporter and nucleotide diphosphatases.

9. The host cell of any one of claims 5 to 8, further expressing one or more exogenous enzymes selected from: a UDP-specific diphosphatase and a GDP-specific diphosphatase.
10. The host cell of any one of claims 1 to 9, obtained by introducing nucleic acid molecules encoding said enzymes.
11. The host cell of claim 10, wherein at least one of the nucleic acid molecules encoding said enzymes is integrated into the host cell chromosome.
12. The host cell of any one of claims 1 to 11, which is selected from the group consisting of *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia sp.*, *Saccharomyces cerevisiae*, *Saccharomyces sp.*, *Hansenula polymorpha*, *Kluyveromyces sp.*, *Candida albicans*, *Aspergillus nidulans*, and *Trichoderma reesei*.
13. The host cell of any one of claims 1 to 12, which is additionally deficient in the activity of one or more enzymes selected from the group consisting of mannosyltransferases and phosphomannosyltransferases.
14. The host cell of claim 13, which does additionally not express an enzyme selected from the group consisting of 1,6 mannosyltransferase; 1,3 mannosyltransferase; and 1,2 mannosyltransferase with respect to the N-glycan.
15. The host cell of any one of claims 1 to 14, wherein the host is an OCH1 mutant of *P. pastoris*.
16. A method for producing the host cell of any one of claims 1 to 15, the method comprising the step of transforming said unicellular or filamentous fungus with a nucleic acid molecule encoding a hybrid enzyme selected to have optimal activity in the ER or Golgi of said host cell, so that said host cell is capable of forming 50 to 100 Mole% Man<sub>5</sub>GlcNAc<sub>2</sub> on a substrate glycoprotein, the hybrid enzyme comprising:
  - (a) an exogenous mannosidase catalytic domain having optimal activity in said ER or Golgi at a pH between 5.1 and 8.0; fused to
  - (b) a cellular targeting signal peptide not normally associated with the catalytic domain of (a), wherein said cellular targeting signal peptide targets said exogenous catalytic domain to said ER or Golgi apparatus.
17. A method for producing a glycoprotein comprising expressing the glycoprotein in a host cell of any one of claims 1 to 15.
18. The method of claim 17, wherein said glycoprotein comprises N-glycans having fewer than six mannose residues.
19. The method of claim 17 or 18, wherein said glycoprotein comprises N-acetylglucosamine.
20. The method of any one of claims 17 to 19, wherein said glycoprotein comprises at least one oligosaccharide branch comprising the structure GlcNAc-Man.
21. The method of any one of claims 17 to 20, wherein said glycoprotein is selected from the group consisting of erythropoietin, interferon- $\alpha$ , interferon- $\beta$ , interferon- $\gamma$ , interferon- $\omega$ , granulocyte-CSF, factor VIII, factor IX, human protein C soluble IgE receptor  $\alpha$ -chain, IgG, IgM; urokinase, chymase, urea trypsin inhibitor, IGF-binding protein, epidermal growth factor, growth hormone-releasing factor, annexin V fusion protein, angiostatin, vascular endothelial growth factor-2, myeloid progenitor inhibitory factor-1 and osteoprotegerin.
22. The method of any one of claims 17 to 21, further comprising the step of isolating the glycoprotein from the host.
23. The method of claim 22, further comprising the step of subjecting the isolated glycoprotein to at least one further glycosylation reaction *in vitro*, subsequent to its isolation from the host.
24. A method for producing a glycoprotein in a host cell that is a unicellular or filamentous fungus that does not display alpha-1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein, the method comprising the steps of:
  - (a) expressing said glycoprotein in a population of said host cells transformed with a DNA library comprising

at least two different genetic constructs both of which comprises a DNA fragment encoding a hybrid enzyme comprising:

- (i) a glycosylation enzyme catalytic domain having optimal activity in said ER or Golgi at a pH between 5.1 and 8.0; fused to
- (ii) a cellular targeting signal peptide not normally associated with the catalytic domain of (i), wherein said cellular targeting signal peptide targets the ER or Golgi apparatus of the host cell; and

(b) selecting the host cell exhibiting a glycoprotein with a desired glycosylation pattern on said glycoprotein.

25. The method of claim 24, wherein the library comprises at least one gene or genetic construct previously subjected to a technique selected from: gene shuffling, *in vitro* mutagenesis, and error-prone polymerase chain reaction.

26. The method of claim 24 or 25, wherein the DNA fragment encoding the glycosylation enzyme catalytic domain has an activity selected from the group consisting of mannosidase, glycosidase, UDP-GlcNAc transferase, glycosyl-transferase selected from GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI; and wherein the cellular targeting signal peptide localizes the enzyme predominantly in a host cell organelle selected from the group consisting of endoplasmic reticulum, cis Golgi, medial Golgi, and trans Golgi.

27. The method of claims 24 or 25, wherein the encoded glycosylation enzyme catalytic domain has a mannosidase activity.

28. The method of claim 27, wherein the host cell further expresses GnT1.

29. The method of any one of claims 24 to 28, wherein the library comprises at least one DNA fragment encoding a wild-type glycosylation enzyme.

30. The method of any one of claims 24 to 29, wherein the selection comprises the step of analyzing a glycosylated protein or isolated N-glycan by one or more methods selected from the group consisting of: (a) mass spectroscopy such as MALDI-TOF-MS; (b) liquid chromatography; (c) characterizing cells using a fluorescence activated cell sorter, spectrophotometer, fluorimeter, or scintillation counter; (d) exposing host cells to a lectin or antibody having a specific affinity for a desired oligosaccharide moiety; and (e) exposing cells to a cytotoxic or radioactive molecule selected from the group consisting of sugars, antibodies and lectins.

31. A transformed host cell produced in the method of any one of claims 16 or 24 to 30.

32. A glycoprotein composition obtainable by any one of the methods of claims 17 to 30, comprising 50 to 100 mole % of  $\text{Man}_5\text{GlcNAc}_2$  converted by GnT I *in vivo* to  $\text{GlcNAcMan}_5\text{GlcNAc}_2$ , said glycoprotein lacking galactose, fucose and terminal sialic acid.

33. A DNA library comprising genetic constructs, the constructs comprising a DNA fragment encoding a cellular targeting signal peptide which targets to the endoplasmic reticulum or Golgi apparatus ligated in-frame with a DNA fragment encoding a glycosylation enzyme or catalytically active fragment thereof having optimal activity in said ER or Golgi at a pH between 5.1 and 8.0, said DNA library including at least two DNA fragments encoding a cellular targeting signal peptide and at least two DNA fragments encoding exogenous glycosylation enzymes.

34. A plurality of host cells comprising a DNA library of claim 33.

## Patentansprüche

1. Wirtszelle, die ein unizellulärer oder filamentöser Pilz ist, und keine alpha-1,6-Mannosyltransferaseaktivität, bezogen auf das N-Glycan auf einem Glycoprotein, aufweist, und die in ihrem Endoplasmatischen Reticulum (ER) oder Golgi-Apparat ein Hybridenzym hat, das ausgewählt ist, um optimale Aktivität im ER oder Golgi der Wirtszelle zu haben, so dass die Wirtszelle 50 bis 100 Mol%  $\text{Man}_5\text{GlcNAc}_2$  auf einem Substrat-Glycoprotein bilden kann, wobei das Hybridenzym umfasst:

(a) eine exogene Mannosidase-katalytische Domäne mit optimaler Aktivität im ER oder Golgi bei einem pH

zwischen 5,1 und 8,0; verbunden mit

(b) einem zellulären Zielsignal-Peptid, das normalerweise nicht mit der katalytischen Domäne von (a) verbunden ist, wobei das zelluläre Zielsignal-Peptid die exogene Mannosidase-katalytische Domäne in das ER oder den Golgi-Apparat bringt.

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2. Wirtszelle nach Anspruch 1, wobei die katalytische Domäne  $\alpha$ -1,2-Mannosidaseaktivität hat.

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3. Wirtszelle nach Anspruch 1 oder 2, wobei die Mannosidase eine  $\alpha$ -1,2-Mannosidase-katalytische Domäne umfasst, die von einer Maus, einem Menschen, *Lepidoptera*, *Aspergillus nidulans*, *Xanthomonas manihotis* oder *Bacillus* sp. stammt.

4. Wirtszelle nach einem der Ansprüche 1 bis 3, wobei die Mannosidase optimale Aktivität bei einem pH zwischen 5,9 und 7,5 hat.

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5. Wirtszelle nach einem der Ansprüche 1 bis 4, die weiterhin ein oder mehrere exogene(s) Enzym(e) exprimiert/exprimieren, ausgewählt aus der Gruppe bestehend aus Mannosidasen, Glycosyltransferasen und Glycosidasen.

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6. Wirtszelle nach Anspruch 5, wobei mindestens ein zusätzliches exogenes Enzym für die Herstellung eines Glycoproteins lokalisiert wird, indem ein Fusionsprotein zwischen einer katalytischen Domäne des exogenen Enzyms und einem zellulären Zielsignal-Peptid gebildet wird, das normalerweise nicht mit der katalytischen Domäne verbunden ist, die es in das Endoplasmatische Reticulum, den frühen, mittleren oder späten Golgi oder in das trans-Golgi Netzwerk der Wirtszelle bringt.

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7. Wirtszelle nach Anspruch 6, wobei die katalytische Domäne von einer Glycosidase oder Glycosyltransferase stammt, ausgewählt aus GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI.

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8. Wirtszelle nach einem der Ansprüche 5 bis 7, die zusätzlich ein oder mehrere exogene(s) Enzym(e) exprimiert/exprimieren, ausgewählt aus der Gruppe bestehend aus UDP-GlcNAc-Transferase, UDP-GlcNAc-Transporter und Nucleotid-Diphosphatasen.

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9. Wirtszelle nach einem der Ansprüche 5 bis 8, die zusätzlich ein oder mehrere exogene(s) Enzym(e) exprimiert/exprimieren, ausgewählt aus: einer UDP-spezifischen Diphosphatase und einer GDP-spezifischen Diphosphatase.

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11. Wirtszelle nach Anspruch 10, wobei mindestens eines der Nucleinsäuremoleküle, welche die Enzyme codieren, in das Wirtszell-Chromosom integriert ist.

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12. Wirtszelle nach einem der Ansprüche 1 bis 11, die ausgewählt ist aus der Gruppe bestehend aus *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia* sp., *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Hansenula polymorpha*, *Kluyveromyces* sp., *Candida Albicans*, *Aspergillus nidulans* und *Trichoderma reesei*.

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13. Wirtszelle nach einem der Ansprüche 1 bis 12, die zusätzlich keine Aktivität eines oder mehrerer Enzyms/Enzyme hat, ausgewählt aus der Gruppe bestehend aus Mannosyltransferasen und Phosphomannosyltransferasen.

14. Wirtszelle nach Anspruch 13, die zusätzlich kein Enzym exprimiert, ausgewählt aus der Gruppe bestehend aus 1,6-Mannosyltransferase; 1,3-Mannosyltransferase; und 1,2-Mannosyltransferase bezogen auf das N-Glycan.

15. Wirtszelle nach einem der Ansprüche 1 bis 14, wobei der Wirt eine OCH1-Mutante von *P. pastoris* ist.

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16. Verfahren zur Herstellung einer Wirtszelle nach einem der Ansprüche 1 bis 15, wobei das Verfahren den Schritt des Transformierens des unizellulären oder filamentösen Pilzes mit einem Nucleinsäuremolekül umfasst, das ein Hybridenzym codiert, das ausgewählt ist, um optimale Aktivität im ER oder Golgi der Wirtszelle zu haben, so dass die Wirtszelle 50 bis 100 Mol% Man<sub>5</sub>GlcNAc<sub>2</sub> auf einem Substrat-Glycoprotein bilden kann, wobei das Hybriden-

zym umfasst:

- (a) eine exogene Mannosidase-katalytische Domäne mit optimaler Aktivität im ER oder Golgi bei einem pH zwischen 5,1 und 8,0; verbunden mit
- (b) einem zellulären Zielsignal-Peptid, dass normalerweise nicht mit der katalytischen Domäne von (a) verbunden ist, wobei das zelluläre Zielsignal-Peptid die exogene Mannosidase-katalytische Domäne in das ER oder den Golgi-Apparat bringt.

17. Verfahren zur Herstellung eines Glycoproteins, umfassend Exprimieren des Glycoproteins in einer Wirtszelle nach einem der Ansprüche 1 bis 15.

18. Verfahren nach Anspruch 17, wobei das Glycoprotein N-Glycane mit weniger als sechs Mannoseresten umfasst.

19. Verfahren nach Anspruch 17 oder 18, wobei das Glycoprotein N-Acetylglucosamin umfasst.

20. Verfahren nach einem der Ansprüche 17 bis 19, wobei das Glycoprotein mindestens eine Oligosaccharid-Verzweigung umfasst, welche die Struktur GlcNAc-Man umfasst.

21. Verfahren nach einem der Ansprüche 17 bis 20, wobei das Glycoprotein ausgewählt ist aus der Gruppe bestehend aus Erythropoietin, Interferon- $\alpha$ , Interferon- $\beta$ , Interferon- $\gamma$ , Interferon- $\omega$ , Granulocyten-CSF, Faktor VIII, Faktor IX, menschliches Protein C, löslicher IgE-Rezeptor- $\alpha$ -Kette, IgG, IgM, Urokinase, Chymase, Hamstoff-Trypsin-Inhibitor, IGF-bindendem Protein, epidermalem Wachstumsfaktor, Wachstumshormonfreisetzendem Faktor, Annexin V-Fusionsprotein, Angiostatin, vaskulärem endotheliale Wachstumsfaktor-2, myeloidem Vorläufer-Inhibitor-Faktor-1 und Osteoprotegerin.

22. Verfahren nach einem der Ansprüche 17 bis 21, weiterhin umfassend den Schritt des Erntens des Glycoproteins vom Wirt.

23. Verfahren nach Anspruch 22, weiterhin umfassend den Schritt das geerntete Glycoprotein mindestens einer Glycosilierungsreaktion *in vitro* zu unterziehen, im Anschluss an das Ernten vom Wirt.

24. Verfahren zur Herstellung eines Glycoproteins in einer Wirtszelle, die ein unizellulärer oder filamentöser Pilz ist, und die keine  $\alpha$ -1,6-Mannosyltransferaseaktivität im Bezug auf das N-Glycan auf einem Glycoprotein aufweist, wobei das Verfahren die Schritte umfasst:

- (a) Exprimieren des Glycoproteins in einer Population von Wirtszellen, die mit einer DNA-Bibliothek transformiert sind, die mindestens zwei verschiedene genetische Konstrukte umfasst, wobei beide ein DNA-Fragment umfassen, das ein Hybridenzym codiert, umfassend:

- (i) eine Glycosilierungsenzym-katalytische Domäne mit optimaler Aktivität im ER oder Golgi bei einem pH zwischen 5,1 und 8,0; verbunden mit
- (ii) einem zellulären Zielsignal-Peptid, das normalerweise nicht mit der katalytischen Domäne von (i) verbunden ist, wobei das zelluläre Zielsignal-Peptid auf das ER oder den Golgi-Apparat der Wirtszelle abzielt; und

- (b) Auswählen der Wirtszelle, die ein Glycoprotein mit einem gewünschten Glycosilierungsmuster auf dem Glycoprotein zeigt.

25. Verfahren nach Anspruch 24, wobei die Bibliothek mindestens ein Gen oder genetisches Konstrukt umfasst, das vorher einer Technik unterzogen wurde, ausgewählt aus: Gene-Shuffling, *in vitro*-Mutagenese und fehleranfälliger Polymerase-Ketten-Reaktion (error-prone polymerase chain reaction).

26. Verfahren nach Anspruch 24 oder 25, wobei das DNA-Fragment, das die Glycosilierungsenzym-katalytische Domäne codiert, eine Aktivität hat, ausgewählt aus der Gruppe bestehend aus Mannosidase, Glycosidase, UDP-GlcNAc-Transferase, Glycosyltransferase, ausgewählt aus GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI; und wobei das zelluläre Zielsignal-Peptid das Enzym vorwiegend in einem Wirtszell-Organell lokalisiert, ausgewählt aus der Gruppe bestehend aus endoplasmatischem Reticulum, cis-Golgi, medium-Golgi, und trans-Golgi.

27. Verfahren nach Anspruch 24 oder 25, wobei die codierte Glycosylierungsenzymkatalytische Domäne Mannosidaseaktivität hat.

28. Verfahren nach Anspruch 27, wobei die Wirtszelle weiterhin GnT I exprimiert.

29. Verfahren nach einem der Ansprüche 24 bis 28, wobei die Bibliothek mindestens ein DNA-Fragment umfasst, das ein Wildtyp-Glycosylierungsenzym codiert.

30. Verfahren nach einem der Ansprüche 24 bis 29, wobei das Auswählen den Schritt des Untersuchens des glycosylierten Proteins oder isolierten N-Glycans anhand eines oder mehrerer Verfahren umfasst, ausgewählt aus der Gruppe bestehend aus: (a) Massenspektroskopie, wie z. B. MALDI-TOF-MS; (b) Flüssigkeitschromatographie; (c) Charakterisieren von Zellen durch Verwenden eines Fluoreszenz-aktivierten Zell-Sortierers, eines Spektrophotometers, Fluorimeters oder Szintillationszählers; (d) In-Kontakt-bringen der Wirtszellen mit einem Lektin oder Antikörper mit einer spezifischen Affinität für einen gewünschten Oligosaccharid-Rest; und (e) In-Kontakt-bringen der Zellen mit einem cytotoxischen oder radioaktiven Molekül, ausgewählt aus der Gruppe bestehend aus Zuckern, Antikörpern und Lektinen.

31. Transformierte Wirtszelle, hergestellt in dem Verfahren nach einem der Ansprüche 16 oder 24 bis 30.

32. Glycoprotein-Zusammensetzung, erhältlich nach einem der Verfahren der Ansprüche 17 bis 30, umfassend 50 bis 100 Mol% von  $\text{Man}_5\text{GlcNAc}_2$ , in vivo durch GnT I zu  $\text{GlcNAcMan}_5\text{GlcNAc}_2$  umgesetzt, wobei dem Glycoprotein Galactose, Fucose und terminale Sialinsäure fehlen.

33. DNA-Bibliothek, umfassend genetische Konstrukte, wobei die Konstrukte ein DNA-Fragment umfassen, das ein zelluläres Zielsignal-Peptid codiert, das in das Endoplasmatische Reticulum oder den Golgi-Apparat abzielt, und das im Leserahmen mit einem DNA-Fragment verbunden ist, das ein Glycosylierungsenzym oder ein katalytisch aktives Fragment davon codiert, welches optimale Aktivität im ER oder Golgi bei einem pH zwischen 5,1 und 8,0 hat, wobei die DNA-Bibliothek mindestens zwei DNA-Fragmente, die ein zelluläres Zielsignal-Peptid codieren, und mindestens zwei DNA-Fragmente, die exogene Glycosylierungsenzyme codieren, enthält.

34. Vielzahl von Wirtszellen, umfassend eine DNA-Bibliothek nach Anspruch 33.

## Revendications

1. Cellule hôte qui est un champignon unicellulaire ou filamentaire qui ne présente pas l'activité d'alpha-1,6 mannosyltransférase par rapport au N-glycane sur une glycoprotéine, ayant dans son réticulum endoplasmique (RE) ou appareil de Golgi une enzyme hybride sélectionnée pour avoir une activité optimale dans RE ou Golgi de ladite cellule hôte de façon que ladite cellule hôte soit capable de former 50 à 100 Mole% de  $\text{Man}_5\text{GlcNAc}_2$  sur un glycoprotéine de substrat, l'enzyme hybride comprenant:

(a) un domaine catalytique de mannosidase exogène ayant une activité optimale dans ledit RE ou Golgi à un pH compris entre 5,1 et 8,0; fusionné à

(b) un peptide signal de ciblage cellulaire qui n'est pas normalement associé au domaine catalytique de (a), où ledit peptide signal de ciblage cellulaire cible ledit domaine catalytique de mannosidase exogène audit RE ou appareil de Golgi.

2. Cellule hôte de la revendication 1, où ledit domaine catalytique a une activité de  $\alpha$ -1,2-mannosidase.

3. Cellule hôte de la revendication 1 ou 2, où ladite mannosidase comprend un domaine catalytique de  $\alpha$ -1,2-mannosidase dérivé de la souris, de l'humain, de *Lepidoptera*, *Aspergillus nidulans*, *Xanthomonas manihotis*, ou *Bacillus* sp.

4. Cellule hôte de l'une quelconque des revendications 1 à 3, où le mannosidase a une activité optimale à un pH compris entre 5,9 et 7,5.

5. Cellule hôte de l'une quelconque des revendications 1 à 4, exprimant de plus une ou plusieurs enzymes exogènes sélectionnées dans le groupe consistant en mannosidases, glycosyltransférases et glycosidases

6. Cellule hôte de la revendication 5, où au moins une enzyme exogène additionnelle pour la production d'une glycoprotéine est localisée en formant une protéine de fusion entre un domaine catalytique de l'enzyme exogène et un peptide signal de ciblage cellulaire qui n'est pas normalement associé au domaine catalytique qui le cible au réticulum endoplasmique, le Golgi précoce médian ou tardif ou le réseau trans Golgi de la cellule hôte.
7. Cellule hôte de la revendication 6, où ledit domaine catalytique provient d'une glycosidase ou d'une glycosyltransférase sélectionnée parmi GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI.
8. Cellule hôte de l'une quelconque des revendications 5 à 7 exprimant de plus une ou plusieurs enzymes exogènes sélectionnées dans le groupe consistant en UDP-GlcNAc transférase, transporteur de UDP-GlcNAc et nucléotide diphosphatases.
9. Cellule hôte de l'une quelconque des revendications 5 à 8 exprimant de plus une ou plusieurs enzymes exogènes sélectionnées parmi: une diphosphatase spécifique de UDP et une diphosphatase spécifique de GDP.
10. Cellule hôte de l'une quelconque des revendications 1 à 9 obtenue par introduction de molécules d'acide nucléique codant pour lesdites enzymes.
11. Cellule hôte de la revendication 10, où au moins l'une des molécules d'acide nucléique codant pour lesdites enzymes est intégrée dans le chromosome de la cellule hôte.
12. Cellule hôte de l'une quelconque des revendications 1 à 11, qui est sélectionnée dans le groupe consistant en *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia sp.*, *Saccharomyces cerevisiae*, *Saccharomyces sp.*, *Hansenula polymorpha*, *Kluyveromyces sp.*, *Candida albicans*, *Aspergillus nidulans*, et *Trichoderma reesei*.
13. Cellule hôte de l'une quelconque des revendications 1 à 12, qui est additionnellement déficiente en activité d'une ou plusieurs enzymes sélectionnées dans le groupe consistant en mannosyltransférases et phosphomannosyltransférases.
14. Cellule hôte de la revendication 13, qui additionnellement n'exprime pas une enzyme sélectionnée dans le groupe consistant en 1,6-mannosyltransférase; 1,3-mannosyltransférase et 1,2-mannosyltransférase par rapport au N-glycane.
15. Cellule hôte de l'une quelconque des revendications 1 à 14, où la hôte est un mutant OCH1 de *P. pastoris*.
16. Méthode de production de la cellule hôte de l'une quelconque des revendications 1 à 15, la méthode comprenant l'étape de transformer ledit champignon unicellulaire ou filamentaire avec une molécule d'acide nucléique codant pour une enzyme hybride sélectionnée pour avoir une activité optimale dans le RE ou le Golgi de ladite cellule hôte, de façon que ladite cellule hôte soit capable de former 50 à 100 Mole% de Man<sub>5</sub>GlcNAc<sub>2</sub> sur une glycoprotéine comme substrat, l'enzyme hybride comprenant:
  - (a) un domaine catalytique de mannosidase exogène ayant une activité optimale dans ledit RE ou Golgi à un pH compris entre 5,1 et 8,0; fusionné à
  - (b) un peptide signal de ciblage cellulaire qui n'est pas normalement associé au domaine catalytique de (a), où ledit peptide signal de ciblage cellulaire cible ledit domaine catalytique exogène audit RE ou appareil de Golgi.
17. Méthode de production d'une glycoprotéine comprenant l'expression de la glycoprotéine dans une cellule hôte de l'une quelconque des revendications 1 à 15.
18. Méthode de la revendication 17, où ladite glycoprotéine comprend des N-glycanes ayant moins de six résidus mannose.
19. Méthode de la revendication 17 ou 18, où ladite glycoprotéine comprend de la N-acétylglucosamine.
20. Méthode de l'une quelconque des revendications 17 à 19, où ladite glycoprotéine comprend au moins une branche

de oligosaccharide comprenant la structure GlcNAc-Man.

21. Méthode de l'une quelconque des revendications 17 à 20, où ladite glycoprotéine est sélectionnée dans le groupe consistant en érythropoïétine, interféron- $\alpha$ , interféron- $\beta$ , interféron- $\gamma$ , interféron- $\omega$ , granulocyte-CSF, facteur VIII, facteur IX, chaîne- $\alpha$  du récepteur de IgE soluble de la protéine C humaine, IgG, IgM, urokinase, chymase, inhibiteur de la trypsine de l'urée, protéine liant IGF, facteur de croissance épidermique, facteur libérant l'hormone de croissance, protéine de fusion d'annexine V, angiostatine, facteur 2 de croissance endothéliale vasculaire, facteur 1 inhibiteur progéniteur de myéloïde et ostéoprotégérine.

22. Méthode de l'une quelconque des revendications 17 à 21, comprenant de plus l'étape d'isoler la glycoprotéine de l'hôte.

23. Méthode de la revendication 22, comprenant de plus l'étape de soumettre la glycoprotéine isolée à au moins une autre réaction de glycosylation *in vitro*, subséquemment à son isolement de l'hôte.

24. Méthode de production d'une glycoprotéine dans une cellule hôte qui est un champignon unicellulaire ou filamentaire qui ne présente pas l'activité d'alpha-1,6 mannosyltransférase par rapport au N-glycane sur une glycoprotéine, la méthode comprenant les étapes de:

(a) exprimer ladite glycoprotéine dans une population desdites cellules hôtes transformées par une bibliothèque d'ADN comprenant au moins deux constructions génétiques différentes, dont toutes deux comprennent un fragment d'ADN codant pour une enzyme hybride comprenant:

- (i) un domaine catalytique d'enzyme de glycosylation ayant une activité optimale dans ledit RE ou Golgi à un pH compris entre 5,1 et 8,0; fusionné à
- (ii) un peptide signal de ciblage cellulaire qui n'est pas normalement associé au domaine catalytique de (i), où ledit peptide signal de ciblage cellulaire cible RE ou l'appareil de Golgi de la cellule hôte; et

(b) sélectionner la cellule hôte présentant une glycoprotéine ayant un motif souhaité de glycosylation sur ladite glycoprotéine.

25. Méthode de la revendication 24, où la bibliothèque comprend au moins un gène ou construction génétique soumis au préalable à une technique sélectionnée parmi: mélange des gènes, mutagenèse *in vitro*, et réaction de chaîne de polymérase sujette à l'erreur.

26. Méthode de la revendication 24 ou 25, où le fragment d'ADN codant pour le domaine catalytique de l'enzyme de glycosylation a une activité sélectionnée dans le groupe consistant en mannosidase, glycosidase, UDP-GlcNAc transférase, glycosyltransférase sélectionnée parmi GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI; et où le peptide signal de ciblage cellulaire localise l'enzyme de manière prédominante dans un organelle de cellule hôte sélectionné dans le groupe consistant en réticulum endoplasmique, Golgi cis, Golgi median, et trans Golgi.

27. Méthode des revendications 24 ou 25, où ledit domaine catalytique d'enzyme de glycosylation codé à une activité de mannosidase.

28. Méthode de la revendication 27, où la cellule hôte exprime de plus GnT I.

29. Méthode de l'une quelconque des revendications 24 à 28, où la bibliothèque comprend au moins un fragment d'ADN codant pour une enzyme de glycosylation du type sauvage.

30. Méthode de l'une quelconque des revendications 24 à 29, où la sélection comprend l'étape d'analyser une protéine glycosylée ou un N-glycane isolé par une ou plusieurs méthodes sélectionnées dans le groupe consistant en: (a) spectroscopie de masse comme MALDI-TOF-SM; (b) chromatographie en phase liquide; (c) caractérisation des cellules en utilisant un trieur de cellules activé à la fluorescence, un spectrophotomètre, un fluorimètre ou un compteur à scintillation; (d) exposer les cellules hôtes à une lectine ou un anticorps ayant une affinité spécifique pour une fraction souhaitée d'oligosaccharide; et (e) exposer les cellules à une molécule cytotoxique ou radioactive sélectionnée dans le groupe consistant en sucres, anticorps et lectines.

31. Cellule hôte transformée produite par la méthode de l'une quelconque des revendications 16 ou 24 à 30.

32. Composition de glycoprotéine pouvant être obtenue par l'une quelconque des méthodes des revendications 17 à 30, comprenant 50 à 100% en moles de  $\text{Man}_5\text{GlcNAc}_2$  converti par GnT I in vivo en  $\text{GlcNAcMan}_5\text{GlcNAc}_2$ , ladite glycoprotéine manquant de galactose, fucose et acide sialique terminal.

33. Bibliothèque d'ADN comprenant des constructions génétiques, les constructions comprenant un fragment d'ADN codant pour un peptide signal de ciblage cellulaire qui se cible au réticulum endoplasmique ou à l'appareil de Golgi ligaturé en cadre avec un fragment d'ADN codant pour une enzyme de glycosylation ou son fragment catalytiquement actif ayant une activité optimale dans ledit RE ou Golgi à un pH compris entre 5,1 et 8,0, ladite bibliothèque d'ADN comprenant au moins deux fragments d'ADN codant pour un peptide signal de ciblage cellulaire et au moins deux fragments d'ADN codant pour des enzymes exogènes de glycosylation.

34. Un certain nombre de cellules hôtes comprenant une bibliothèque d'ADN de la revendication 33.

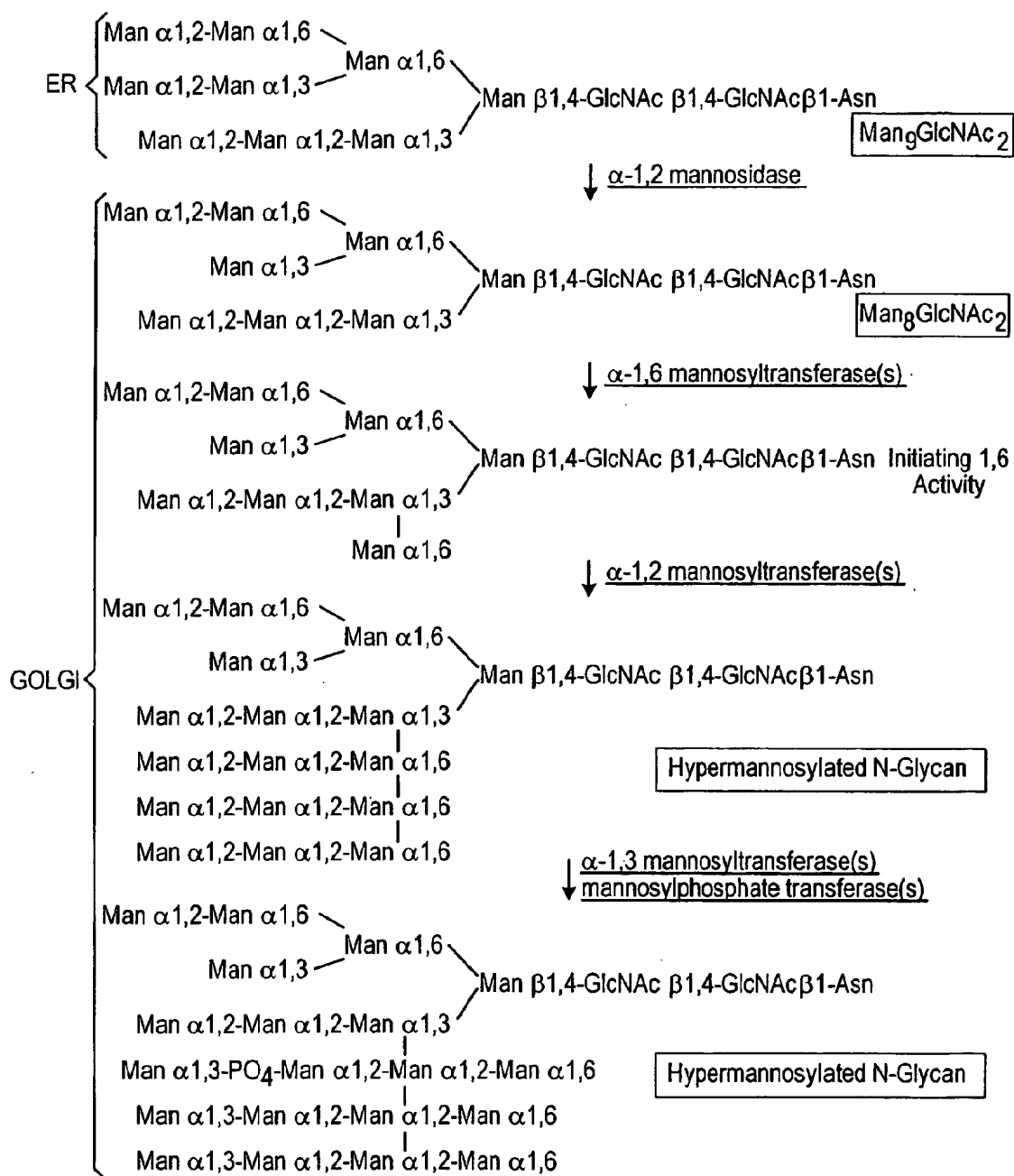


FIG. 1A

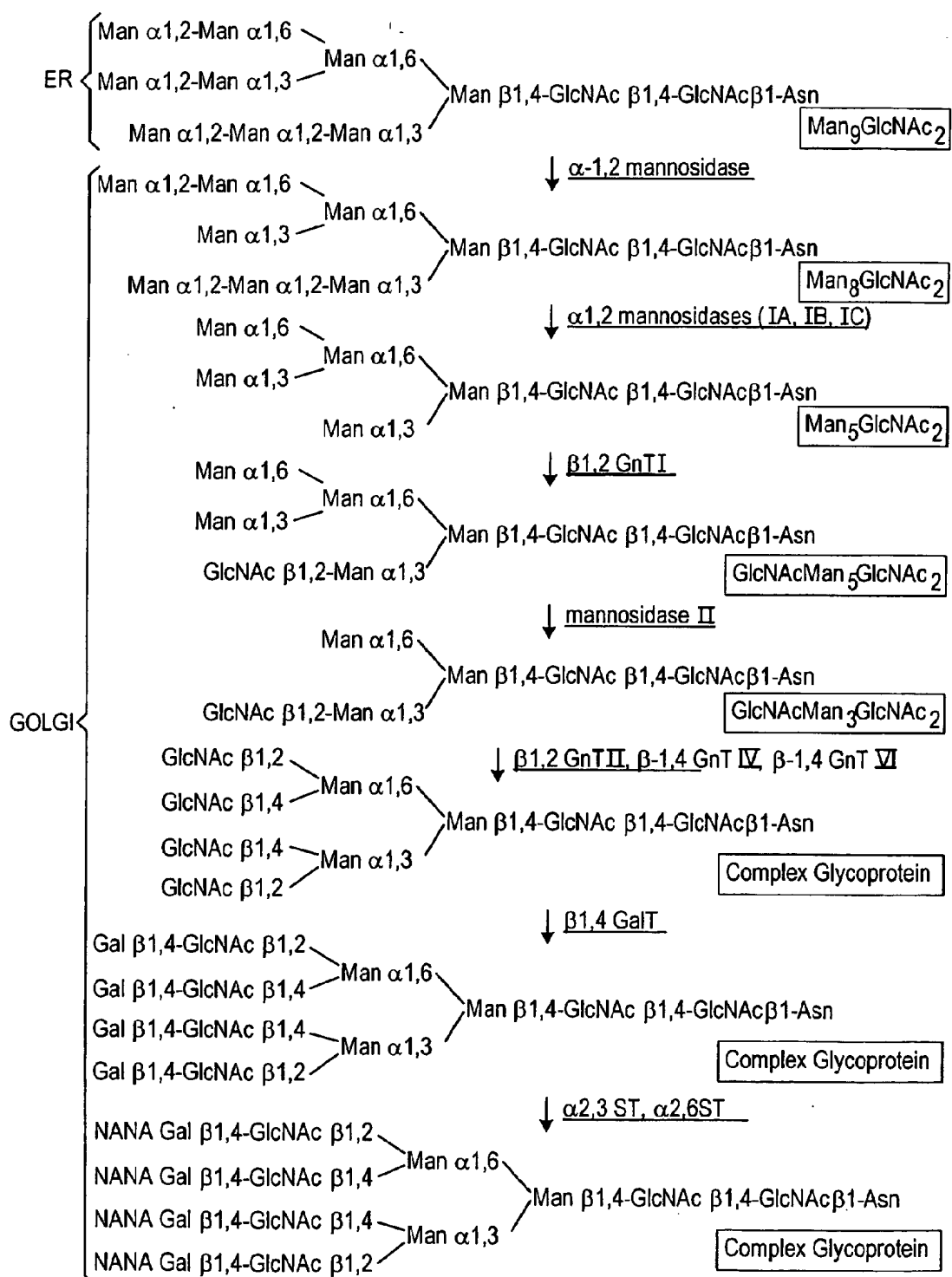


FIG. 1B